DOCTOR OF MEDICINE

A combined clinical and experimental study to investigate a potential model for improved osseointegration in sickle cell bone disease patients with avascular necrosis

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A COMBINED CLINICAL AND EXPERIMENTAL STUDY TO INVESTIGATE A POTENTIAL MODEL FOR IMPROVED OSSEOINTEGRATION IN SICKLE CELL BONE DISEASE PATIENTS WITH AVASCULAR NECROSIS

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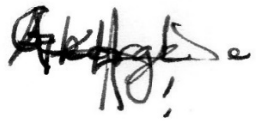
Signed  AKINTUNDE GEORGE 04/06/2019

ABSTRACT

Sickle cell disease (SCD) is an inherited blood disorder, the natural history of which leads to degenerative avascular necrosis of the femoral head. The definitive long-term treatment for this debilitating condition is total hip arthroplasty. It is well established that the failure rate of the implants in these patients is very high and so leads to an increased revision burden. Literature has shown that all mammalian cells express the hypoxic inducible factor (HIF) which in the case of bone cells enhances osseointegration in a hypoxic environment. Within the *in vivo* physiologic environment in sickle cell patients, hypoxia is prevalent due to the sickling process, which leads to bony cellular hypoxia and then avascular necrosis. This research work was aimed at establishing if there was any structural reason for implant loosening due to the shape of the proximal femur in sickle cell disease patients. This study sought also to investigate the role of HIF in improving osseointegration by using an experimental model. It was important to ascertain if this model could give the needed physiologic assurance in the viability of osteoblasts cultured under hypoxic conditions.

The clinical study used radiographic evaluation of proximal femurs of sickle cell disease patients, assessing them against established classifications to ascertain their shape and comparison drawn from osteoarthritic patients. In the experimental study, MG63 osteoblast cells were cultured under true hypoxic conditions (1% oxygen) in the presence of cobalt chloride and using the qualitative polymerase chain reaction (qPCR) process, the expression of HIF was evaluated. Using the same identical conditions as stated above, MG63 cells was further cultured and their physiologic viability was assessed using the Alamar blue assay.

The radiographic study showed clearly that sickle cell disease patients had well defined funnel shaped femurs but less so than the osteoarthritic cohort. This might allow for less press fit femoral component to be implanted and together with having less cortical thickness, there is a risk of occult and overt periprosthetic fractures which may contribute to femoral component loosening. The results from the experimental work confirmed that HIF could be up regulated in MG63 osteoblast cells in true hypoxic conditions made possible in the presence of cobalt chloride and that osteoblasts were viable despite the subnormal physiologic experimental conditions of hypoxia and cobalt chloride under which these cells were cultured.

From the clinical work undertaken, it was concluded that there might be structural reasons for the increased lack of osseointegration in sickle cell disease with femoral hip prosthesis. Furthermore, it was established that the experimental model used to investigate HIF expression and osteoblast cellular viability under hypoxic conditions could be deployed as an effective research tool for improving osseointegration in sickle disease patients with total hip replacement. It can be concluded from this work that cobalt chloride could be an agent for enhancing osseointegration, which is different from its current use as a bearing surface. This model could also be used in improving implant design to help address the increased total hip revision burden seen in these patients.

DEDICATION

I would want to dedicate this work to sickle cell disease patients with total hip replacements who seek to have a better quality of life despite the harsh reality of high prosthetic implant failure.

ACKNOWLEDGEMENTS

I want to thank the Lord Jesus for giving me the strength and grace to do this work. I appreciate His loving kindness towards me and the opportunity He has given me to engage in this research.

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CONTENT PAGE

LIST OF TABLES

LIST OF FIGURES

AIMS AND STRUCTURE OF THESIS

CHAPTER ONE - *LITERATURE REVIEW*

This thesis was aimed at first reviewing sickle cell disease and its bony sequelae of avascular necrosis of the femoral head of which the total hip replacement is the long-term definitive treatment. We reviewed the various type of hip prosthesis fixation modes, mechanism of failure and the problems of high failure rate of hip implants in these patients. This contributes to the high revision burden they experience. The review also aimed at bringing to the fore the role of the hypoxic inducible factor (HIF), a transcription protein needed for osteogenesis in a hypoxic bony environment. This is the suboptimal macro cellular bone marrow environment where the femoral component for THR is implanted in sickle cell disease patients. The review further raised questions on how osseointegration could be improved in this environment. With this in mind, the need to develop experimental models to investigate this was obvious and hence the reason for the *in vitro* model designs and proposals in the subsequent chapters.

CHAPTER TWO - *RADIOLOGIC EVALUATION OF THE PROXIMAL FEMUR IN SICKLE CELL DISEASE PATIENTS*

Having identified the need to explore how to improve osseointegration in a hypoxic environment, we first aimed to establish if the shape of the proximal femur in SCD patients contributed to femoral component implant loosening. This was achieved through a comparative radiologic study of the proximal femur shape in sickle cell disease patients compared with similar osteoarthritic patients. A secondary objective was to also explore the variation in the shape of the proximal femur in these patients and characterize it.

CHAPTER THREE – *THE EXPERIMENTAL STUDY TO INVESTIGATE A POTENTIAL MODEL FOR IMPROVED OSSEOINTEGRATION IN SICKLE CELL BONE DISEASE PATIENTS WITH AVASCULAR NECROSIS*

The need to investigate and improve osseointegration forms part of the basis for successful hip arthroplasty treatment in patients. We therefore sought to explore this physiologic phenomenon by setting up *in vitro* experimental models, which would be able to mimic the hypoxic *in vivo* pathologic environment seen in SCD patients, and see if over expression of HIF was a possibility. This was to be done in the presence of cobalt chloride - a hypoxic mimetic.

CHAPTER FOUR - *INVESTIGATING CELLULAR VIABILITY OF MG63 CELLS WITH COBALT CHLORIDE IN AN HYPOXIC ENVIRONMENT- A PROPOSED IN VITRO MODEL*

We also wanted to explore the viability of osteoblasts in the above - enhanced hypoxic environment. This was investigated through another experimental *in vitro* model with MG63 cells cultured with cobalt chloride. Through this model, the objective of checking the functional potential of osteoblasts was going to be assessed in order to lend some credence to the previous experimental study and somehow authentic its future effectiveness. We hope that this study could form the basis for improved femoral implant design, which can be used in improving osseointegration in these patients.

CHAPTER FIVE- *GENERAL DISCUSSIONS*

We hope with the results of this work, that improvements could be made to the experimental model proposed. This can be seen as the first building blocks to further explore the design of *in vivo* models, which could later form the basis for knowledge transfer into clinical practise for the improvement of hip implant survival in sickle cell disease patients.

CHAPTER SIX - *FUTURE WORK AND PROPECTS*

With the scientific literature being devoid of little evidence into experimental models investigating osseointegration particularly in sickle cell disease patients, this research study may be able to led the way in developing improved osseointegration focused *in vitro* models. These models could aim at improving osseointegration in these sickle cell patients with regards their hip implants and this would reduce significantly their surgical hip revision burden. Furthermore, it is possible that with this work, some ideas targeted at improving hip femoral implant design with regards coating the femoral implant surface with osteogenic proteins such as HIF could be seen in the near future and used in sickle cell disease patients with AVN having total hip replacement surgery.

CHAPTER ONE- THESIS INTRODUCTION

LITERATURE REVIEW

1.0 *INTRODUCTION*

Avascular necrosis (AVN) of the femoral head remains a common complication in sickle cell disease (SCD) patients and this is frequently bilateral. This is caused by infarctions which results from occlusion of the vasculature by red blood cells (erythrocytes) which have changed shape to flow less smoothly in the blood vessels. The red blood cells take the shape similar to a crescent or sickle hence the name of the disease. This makes them less passible through blood vessels compared to normal rounded erythrocytes. In addition, this abnormal shape makes them adhere to other erythrocytes and the vascular endothelial walls causing occlusion, bone marrow ischemia and eventual progression to AVN (Ilyas & Moreau, 2002; Sanders, 2018).

Total hip replacement (THR) surgery is often necessary to treat the degeneration which results from AVN (Hanker & Amstutz, 1988). However, it has been noted that the outcome and survivorship of this procedure in SCD patients is relatively poor in comparison with THR from other aetiologies and the failure of osseointegration is attributed as a major underlying reason (Acurio & Friedman, 1992; Amstutz & Le Duff, 2016; Bishop, Roberson, Eckman, & Fleming, 1988). There is still robust debate out there in the orthopaedic surgical community on the choice between using cemented or uncemented implants or even still hybrid (combination of cemented and uncemented components) in the definitive treatment of AVN in SCD. However there is emerging current literature that has shown some promising results with the use of uncemented prosthesis (Gulati et al., 2015) which this review would further elaborate on.

This chapter seeks to outline the sickle cell bone disease epidemiology, pathophysiology, the treatment and recognized complication of implant loosening which these patients have. It also seeks to shine the light on the physiologic phenomenon of osseointegration and how the study of this has influenced orthopaedic implant design. It identifies some of the various *in-vitro* studies used to investigate this phenomenon and how any of these studies have affected hip arthroplasty surgery in sickle cell disease patients.

Many studies were carried out to evaluate the various cellular and molecular processes involved in osteogenesis. Angiogenesis and bone formation work closely together in this (Wang et al., 2007). The hypoxia inducible factor (HIF) which is stimulated in tissue hypoxia triggers a cascade of molecular processes that helps manage this physiologic deficiency (Keith & Simon, 2007; Lampert, Kutscher, Stark, & Finkenzeller, 2016). However, there remains a paucity of knowledge concerning how sickle cell bone pathology in particular avascular necrosis can be altered when it comes to osseointegration at the molecular level. The Hypoxia Inducible Factor has been identified as a key factor in mediating how cells adapt to molecular oxygen levels.

This review further elucidates the physiology of the hypoxic inducible factor with its various pathways and establishes what role this factor could play in altering the pathophysiology of avascular necrosis caused by sickle cell disease. It seeks to establish certain research methodology frameworks in exploring how osseointegration can be improved in these patients with prosthetic implants especially total hip replacements.

One major purpose to this literature review is to highlight questions with regards the paucity of experimental models in the current literature that will help form the basis for research to improve osseointegration in SCD patients. With considerable prior work having been undertaken to understand the mechanism of microcellular bony hypoxia, the current study set out to bring to the fore the need to investigate further potential *in vitro* experimental models which will mimic the *in vivo* pathologic setting in sickle cell avascular necrosis. This may help lead to creating some foundation building blocks that could help form the basis for directed research at improving osseointegration in sickle cell disease patients.

1.1 *SICKLE CELL BONE DISEASE*

Sickle-cell disease is an inherited blood condition common among, but not confined to, peoples of Equatorial African ancestry. The gene for sickle haemoglobin (HbS) results in the substitution of valine for glutamic acid normally present at the sixth position from the amino terminus of the chain of haemoglobin. It is acquired by inheriting abnormal genes from both parents, the combination giving rise to different forms of sickle cell disease. Most common at birth is homozygous sickle-cell (SS) disease, also called sickle-cell anaemia, in which the HbS gene is inherited from both parents (Serjeant, 1997).

Sickle-cell disease (SCD) encompasses various combinations of abnormal haemoglobin genes that include at least one copy of the gene for haemoglobin S paired with another structural β-chain haemoglobin variant or β-thalassemia gene.

1.1.1 *EPIDEMIOLOGY*

People living in Africa have the highest burden of sickle-cell disease, predominantly due to four types of abnormal haemoglobin combinations: haemoglobin SS (sickle-cell anaemia), haemoglobin SC, haemoglobin Sβ+ thalassemia, and haemoglobin Sβ0 thalassemia. The sickle cell trait is widespread throughout Africa with low frequencies (<1%–2%) in the north and south of the continent and high but variable frequencies throughout much of equatorial Africa (Nagel et al., 1985; Pagnier et al., 1984). Some estimated 300,000- 400,000 babies are born with sickle cell anaemia (SCA) worldwide every year. Approximately 75% of these births occur in sub-Saharan Africa (Kato et al., 2018; Macharia et al., 2018; D. C. Rees, 2014) (*See Figure 1.1)*. Population estimates in the United States suggests that approximately 100,000 people have the disease. It is estimated that three countries in the world account for the majority of babies with sickle cell disease. These countries are Nigeria, Democratic Republic of Congo and India. Unfortunately the mortality of children under 5 years with the condition can be as high as 90% (Grosse et al., 2011). Demographic projections estimate that by 2050, the number of newborn babies with SCA worldwide will increase by a third and in the above-mentioned countries there would an increase of 30% (Piel, Hay, Gupta, Weatherall, & Williams, 2013; Ware, de Montalembert, Tshilolo, & Abboud, 2017).

Importantly for Europe, there are scarce non-current data to the best of our knowledge for prevalence of sickle cell disease in the region. However in 2010, it was reported that about over 3,000 children are born with SCD every year in the region (Piel et al., 2010). This figure could be unreliable bearing in mind the significant migration into Europe in recent years and substantial number of children are being born in areas where SCD is considered rare (for example northern and western Europe) (Thein, Igbineweka, & Thein, 2017). The total number of patients with SCD among migrants in Germany was estimated at 2,106 in 2007 and 3,216 in 2015, which is an increase of 60% (Kunz et al., 2017). It is also worth noting that in a sickle cell multicenter study carried out in both United Kingdom and France, retrospective data showed a 6% mortality rate in SCD patients (Perronne et al., 2002).

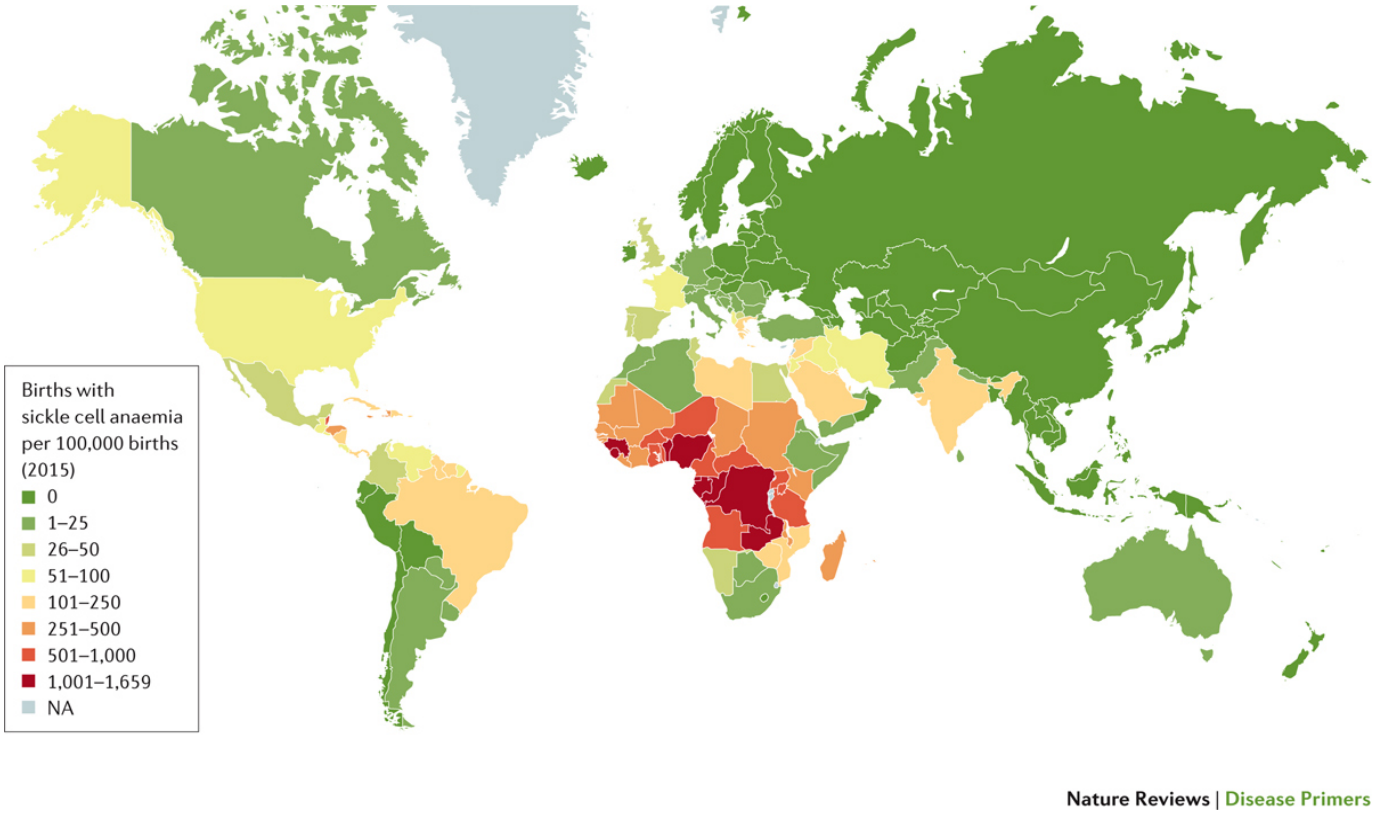

Figure 1.1 *Map of the estimated numbers of births with sickle cell anaemia Reference-. Kato, G. J. et al. (2018)*

1.1.2 *EPIDEMIOLOGIC PROFILE FOR AVASCULAR NECROSIS IN SICKLE CELL DISEASE*

The femoral head is the most commonly affected site for AVN followed by the humeral head. Studies in the literature reported the incidence of osteonecrosis of the femoral head (ONFH) in SCD could vary from 3%-50% (Issa et al., 2013). The co-operative study of SCD reported that the estimated age for the diagnosis for AVN was 28 years and age-specific prevalence rate was highest in patients who were over 45 years of age (34.9%) (Thein et al., 2017). In contrast, the prevalence among patients under 25 years was approximately 6%.
Furthermore, it is important to note that AVN is a complication associated with age, with incidence higher in the older patient. Some studies have shown that the prevalence of osteonecrosis of the femoral head is approximately 3% in patients under 15 years and could be as high as 50% in over 35 (Almeida-Matos, Carrasco, Lisle, & Castelar, 2016). Ortiguera *et al* in their series noted that the 50% revision rate in patients who had THR was due to osteonecrosis. However, Johannsson *et al* in their study postulated that osteonecrosis in itself is not a predictor of high failure rate in THR but highlighted that the high revision rate in patients was associated with SCD (Johannson et al., 2011; Ortiguera, Pulliam, & Cabanela, 1999)

1.2 ***PATHOPHYSIOLOGY***

The most important pathophysiological event in sickle cell anaemia that explains most of its clinical manifestations is vascular occlusion. This involves both the micro- and macrovasculature. Osteonecrosis is caused by the interruption of blood flow, limiting the delivery of oxygen and nutrients, and leading to necrosis of bony tissue. (Ballas, 2002; Chughtai et al., 2017). Haemoglobin polymerization leading to erythrocyte rigidity and vasocculsion, is central to the pathophysiology of this disease (David C. Rees, Williams, & Gladwin, 2010). Kaul *et al* noted in their review of the pathophysiology of vascular obstruction in sickle cell syndromes that red cell destruction leads to enhanced cell vascular adhesion which invariably led to more vasocculsion *(Figure 1.2A and 1.2B).*
Bone involvement is the commonest clinical manifestation of sickle cell disease both in the acute setting such as painful vasocclusive crisis and as a source of chronic, progressive disability such as avascular necrosis (Almeida & Roberts, 2005; Kaul, Fabry, & Nagel, 1996). AVN of the femoral head is one of the significant complications affecting the musculoskeletal system in patients with sickle cell haemoglobinopathy. The reported incidence of femoral head necrosis varies from less than 10% to more than 30% (Ellis & Chaudhuri, 2007;

Mavrogenis, Dimitriou, Parvizi, & Babis, 2009). In many patients, both hips and other bones are affected. The pathophysiology of osteonecrosis in sickle cell disease seems to differ from osteonecrosis because of other aetiologies. When magnetic resonance imaging (MRI) is used to quantify lesions in AVN of the femoral head, the lesions seen in sickle cell disease are larger than those seen in osteonecrosis due to other aetiologies (Malizos et al., 2007). In osteonecrosis from other causes, the localization and size of the lesions is directly related to the mechanical stresses on the femoral head. The larger size and wider distribution of the lesion in sickle cell disease is due to variety of independent factors which result in vascular occlusion (Almeida & Roberts, 2005).

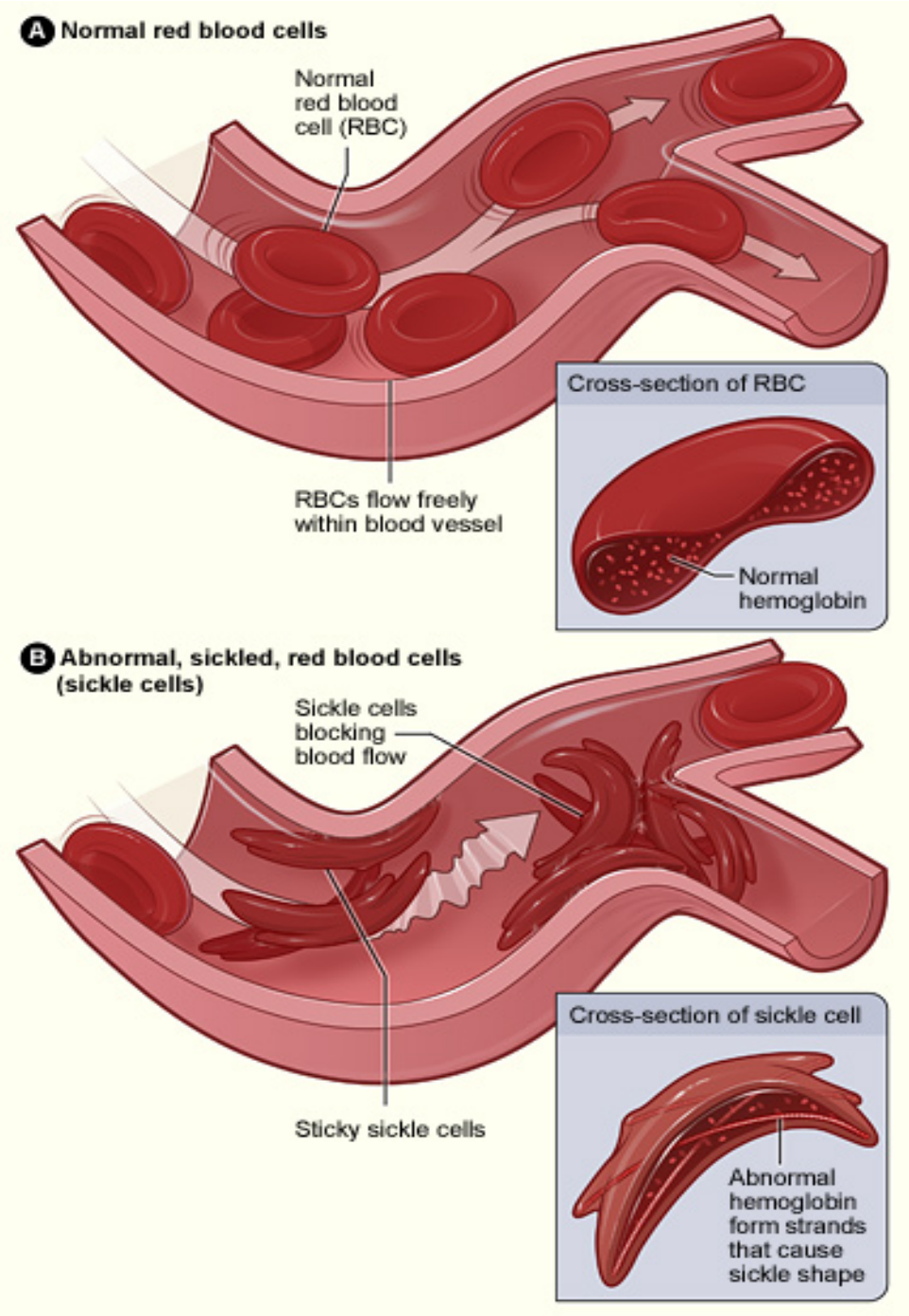

Figure 1.2 (A) shows normal red blood cells flowing freely in a blood vessel. The inset image shows a cross-section of a normal red blood cell with normal haemoglobin.

Figure 1.2 (B) shows abnormal, sickled red blood cells blocking blood flow in a blood vessel. The inset image shows a cross-section of a sickle cell with abnormal (sickle) haemoglobin forming abnormal strands.

Reference: Source- http://www.nhlbi.nih.gov/health/health-topics/topics/sca/ The National Heart, Lung, and Blood Institute (NHLBI)-Image in public domain

1.3 *TOTAL HIP REPLACEMENT FOR SICKLE CELL DISEASE*

As noted earlier, AVN of the femoral head is a common consequence of vasocclusive attacks. The small blood vessels of the femoral head with its specific blood supply and lack of collateral circulation are particularly liable to occlusion by sickled cells. Local thrombosis gives a further reduction of the oxygen tension, resulting in increased sickling. This vicious cycle of continued hypoxia and sickling eventually produces infarction, necrosis, femoral head collapse and joint degeneration and destruction *(Figure 1A).* Hip symptoms are commonly seen in the second and third decades in these SCD patients. Hip replacement arthroplasty is becoming a more frequent operation in the management of those patients who have passed the stage of more conservative surgery. Decision for surgery is based on severity of pain and functional disability. Advances in medical treatment have led to improved life expectancy in sickle cell patients, which in turn lead to an increasing number of patients requiring Total Hip Replacement (THR) (Acurio & Friedman, 1992; Al-Mousawi et al., 2002; Meier, 2018).

1.3.1 BASIS FOR IMPLANT FIXATION AND FAILURE

As a preamble, it would be ideal to review briefly the biomechanical basis for which cemented and uncemented implants achieve bonding in hip arthroplasty. Long-term fixation of component parts of prosthetic joint replacement is accomplished with cement polymethylmethacrylate (PMMA) or uncemented fixation. The cemented fixation is achieved with static mechanical micro-interlock of the PMMA with the endosteal bone. The cement may undergo fatigue and then micro fractures could occur with cyclic loading. As cement cannot remodel, the implant will ultimately loosen. Cemented cups have been noted to have a higher rate of failure especially in younger patients (of which SCD patients belong to this demographic group). The acetabular cup is positioned at an angle relative to the longitudinal axis of the leg and this creates shear tension forces at the cement-bone interface. PMMA is weaker in tension and stronger in compression and hence the lower femoral failure rate of cemented femoral stems as compared to the cups (Miller. M.D, 2016). However, young active patients have increased failure over time. Some autopsy studies have shown that long-term failure of the fixation of cemented femoral components was primarily mechanical, starting with debonding at the interface between the cement and the prosthesis, and continuing as slowly developing fractures in the cement mantle (Jasty et al., 1991) but modern implant design and cementation techniques have helped to improve survival rates in these cases.

The uncemented implants achieve bonding via biologic fixation. This is done using prosthesis fabricated with metal pores. Bone grows into the porous structure stabilizing the implant unto bone. Successful bone ingrowth is based on optimal pore size (50-150μm), optimal metal porosity, minimal implant micro motion, and cortical contact with bone. Some of these factors are ensured with the use of modern uncemented implants hence their use in younger patients including SCD patients with AVN. The initial rigid implant fixation to host is required for long term osseointegration (Ballas, 2002; Miller. M.D, 2016; Ramachandran Manoj, 2006). Despite the limitation and complications of uncemented implants which include proximal-distal mismatch, non-ideal load transfer, loss of bone, difficulties with minimally invasive surgery, periprosthetic fractures (Patel, Smith, Woodward, & Stulberg, 2012), their preferred use in younger patients has formed their functional use in SCD patients. Therefore, for this review we have focused on the investigation of osseointegration and aim to propose an experimental model to help improve this physiologic phenomenon in these patients.

1.3.2 *CEMENTED VERSUS UNCEMENTED IMPLANTS*

Early published series of THR among SCD patients reported high complications and failure rate ranging between 18% and 100%. Improvement in intra and postoperative care and especially of the implant design has resulted in better outcome in reports within the last decade (Al Omran, 2013). Clarke et al noted in their series of cemented THR cases, a high morbidity in sickle cell disease patients due to implant loosening (Clarke, Jinnah, Brooker, & Michaelson, 1989). Other complications (intra-operative and immediate post-operative) in these patients include, deep infection, acetabular protrusion and femoral shaft injury (Al-Mousawi et al., 2002; Bishop et al., 1988). Acurio *et al* noted a 40% revision rate at 7.5 years due to either radiologic and/or symptomatic implant loosening in cemented implants (Acurio & Friedman, 1992) *(See Table 1.3Aa).* Having noted earlier that sickle cell patients with THR are young, the revision rate associated with total hip arthroplasty with cement in young patients has been reported to range from 5% to 49% over follow-up periods of sixteen to twenty-three years. The reported rates of revision of acetabular components in young patients have ranged from 1% to 40% at fifteen years postoperatively (Callaghan, Forest, Olejniczak, Goetz, & Johnston, 1998; Dorr, Kane, & Conaty, 1994; Smith, Estok, & Harris, 2000). These rates are quite high; however, these studies were carried out before the advent of modern cementing techniques that included the use of a distal cement plug to occlude the canal, washing and drying the canal with retrograde filling using a cement gun.

Pressurization then achieved using a proximal seal and the injection of further cement once the canal had been filled (Maggs et al., 2016). It is documented in the literature that the use of cement is likely to cause thermal necrosis of already infarcted bone contributing to higher incidence of infection and loosening. However, Hernigou *et al* (Hernigou, Bachir, & Galacteros, 1993; Hernigou et al., 2008) reported long-term results for a large series of patients with SCD who underwent cemented THR and had promising results compared to others *(Table 1.3a).* They postulated that low rate of implant loosening seen in their results was associated with the use of the "French Paradox" technique. This involves using a largest possible rectangular canal filling titanium alloy stem without trying to obtain a continuous cement mantle. They explained that the rectangular cross section and direct load transfer to the bone by close cortical contact provided intrinsic stability within the femur which in turn might have protected the cement mantle (Langlais, Kerboull, Sedel, & Ling, 2003). It can therefore be suggested that cemented THR could be used with caution in these patients because loosening is a major concern for both femoral and acetabular components (Ilyas, Alrumaih, & Rabbani, 2018).

Table 1.3a *Literature Review for studies with cemented Total Hip Arthroplasty for Sickle Cell Disease. Reference:(Farook, Awogbade, Somasundaram, Reichert, & Li, 2018)*

Author/year of study	No. of hips	Mean follow up (years)	PJI rates (%)	Aseptic failure acetabulum	Aseptic failure femur (%)	Revision Rate (%)
(Al-Mousawi et al., 2002)	37	9.5	2	20		20
(Hernigou et al., 2008)	312	13	3	8	5	13.5
(Al Omran, 2013)	46	12	2.2	36.9	21.7	60.8

Various reasons have been postulated for the high rate of implant loosening with uncemented prostheses. First, earlier series published showing poor results could be due to lack of now improved implant design in uncemented implants *(See Table 1.3b).* Also, due to the chronic anaemia seen in SCD patients, the medullary cavity widens and there is thinning of the cortices. This usually occurs in the metaphyseal region of long bones such as the proximal femur producing weakness, increased chance of fracture and a less than optimal environment for a femoral prosthesis. Bone marrow hyperplasia and repeated infarctions

produces a less favorable biologic environment for the prosthesis leading to high failure rate of acetabular and femoral components. It has also been suggested that lack of bone in-growth (osseointegration) in these patients could contribute to the high loosening rate in them but this is yet to be verified by autopsy studies (Acurio & Friedman, 1992; Al Omran, 2013). Histologic along with a concurrent magnetic resonance imaging study did conclude that acetabular necrosis might be an accompaniment to aseptic necrosis of the femoral head. Further work is required to assess its importance in premature loosening of the acetabular element of total hip arthroplasty (Fink, Assheuer, Enderle, Schneider, & Ruther, 1997). Furthermore, it is worth noting that large cohort studies have shown that the mean age of patients having THR for AVN in SCD is 36 years and so high level of activity in these patients does contribute to increased implant loosening rate. Seeing that these patients are generally young, biologic fixation with modern uncemented implants could be a more attractive option. More recent published large cohort studies with long term follow up in SCD with uncemented THR implants have shown very encouraging results so far (Ilyas et al., 2018; Jack, Howard, Aziz, Kesse-Adu, & Bankes, 2016; Katchy, Anyaehie, Nwadinigwe, & Eyichukwu, 2018).

Table 1.3b *Literature review for studies with uncemented Total Hip Arthroplasty for Sickle Cell Disease. Reference-(Farook et al., 2018)*

Author/year of study	No. of hips	Mean follow up (years)	PJI rates (%)	Aseptic failure acetabulum	Aseptic failure femur (%)	Revision Rate (%)
(Al Omran, 2013)	90	5	1.1	21.1		22.2
(Issa et al., 2013)	42	7.5	4.7	5	5	11.9
(Jack et al., 2016)	52	5.1	1	0	0	3.8
(Gulati et al., 2015)	50	3.8	0	0	0	0
(Azam & Sadat-Ali, 2016)	84	7.5	3.5	8.3		9.5
(Ilyas et al., 2018)	133	14.5	3.76	0.75	0.75	8.2

1.3.3 - *HYBRID IMPLANTS FOR SICKLE CELL DISEASE PATIENTS*

Farook et al noted in their review that hybrid implants (combination of cemented and uncemented THR components) could be an option in these patients. As is the case with both cemented and uncemented implants, it has been noted that there were much higher revision rates in earlier studies *(See Table 1.3c).* In their own study of 34 patients who underwent THR with hybrid implants, there was a revision rate of 17.6 % as compared to an earlier similar study with 35 patients but with a revision rate 40-59% (Acurio & Friedman, 1992; Farook et al., 2018). This difference could be due to the better design of cemented implants and the use of improved cementing techniques**.** Kim et al concluded in their series of young patients with OFN that contemporary uncemented cups and cemented or uncemented stems provide durable long-term fixation and substantial pain relief well into the second decade after the operation. Although the long-term fixation of the acetabular metallic shell and cemented or uncemented femoral stem was outstanding, wear and periacetabular osteolysis constitute the major challenge associated with these contemporary total hip arthroplasties with or without cement in young patients with osteonecrosis of the femoral head (Y. H. Kim, Kim, Park, & Joo, 2011).

Table 1.3c- *Literature Review- for studies with a mix of uncemented, cemented and hybrid For Sickle Cell Disease. Reference:(Farook et al., 2018)*

Author/year of study	No. of hips	Mean follow up (years)	PJI rates (%)	Aseptic failure acetabulum	Aseptic failure femur (%)	Revision Rate (%)
(Acurio & Friedman, 1992)	35	8.6	20	46	46	40 (Cemented-59 Uncemented-22)
(Clarke et al., 1989)	27	5.5	3	N/A	N/A	59
(Hanker & Amstutz, 1988)	16	6.5	2	25	25	63
(Farook et al., 2018)	34	10.5	5.8	11.7	0	17.6

1.4 *OSSEOINTEGRATION*

Osseointegration refers to a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant. It can also be referred to as a direct bone-to-metal interface without interposition of non-bony tissue. Branemark who first described this phenomenon showed that titanium implants could become permanently incorporated within bone that is, the living bone could become so fused with the titanium oxide layer of the implant that the two could not be separated without fracture (Shubayev, Branemark, Steinauer, & Myers, 2004). An implant is considered as osseointegrated when there is no progressive relative movement between the implant and the bone with which it has direct contact. Essentially, the process of osseointegration reflects an anchorage mechanism whereby non-vital components can be reliably incorporated into living bone and which persist under all normal conditions of loading. The biology of osseointegration involves the events that are similar to those occurring during bone healing. Various cell types, growth factors and cytokines are involved and interact throughout the stages of osseointegration, including inflammation, vascularization, bone formation and ultimately bone remodeling (Dimitriou & Babis, 2007).

Factors may enhance or inhibit osseointegration. Factors, which promote osseointegration, include implant-related factors such as implant design and chemical composition, topography of the implant surface, material, shape, length, diameter, implant surface treatment and coatings, the status of the host bone bed and its intrinsic healing potential. Others include the mechanical stability and loading conditions applied on the implant, the use of adjuvant treatments such as bone grafting, osteogenic biological coatings and biophysical stimulation and pharmacological agents such as simvastatin and bisphosphonates. It is important to achieve the best possible implant osseointegration into the adjacent bone and therefore to ensure long-term implant stability. For this purpose, various pharmacological, biological or biophysical modalities have been developed, such as bone grafting materials, pharmacological agents, growth factors and bone morphogenetic proteins. Biophysical stimulation of osseointegration includes two non-invasive and safe methods that have were initially developed to enhance fracture healing: pulsed electromagnetic fields (PEMFs) and low intensity impulse ultrasounds (LIPUS), for which most studies confirm their beneficial effects. Factors, which inhibit osseointegration, include excessive implant mobility and micro motion, inappropriate porosity of the porous coating of the implant, radiation therapy and pharmacological agents such as cyclosporine A (Dimitriou & Babis, 2007; Mavrogenis et al., 2009).

There have been studies looking at osseointegration in relation to biomaterials, and implant surgery. Despite advances in total hip arthroplasty, failure of acetabular cups in total hip arthroplasty remains a concern and more so when those affected are sickle cell disease patients. Kalia et al noted in their study that Osseointegration improved with acetabular cups sprayed with bone marrow stromal cells (Kalia et al., 2009). Other studies have concluded that other biomaterials such as zirconium had similar biocompatibility and osseointegration to titanium implants (Moller et al., 2012). Alternatively, surface topography modifications and physiochemical treatments of surfaces to achieve an enhanced bone implant interface formation using biochemical methods offer an alternative path. These functionalization approaches require a deeper understanding of biology and biochemistry of the host tissue at the interface in terms of mechanisms by which cells adhere to surfaces, the role of biomolecules, functional peptide sequences and extracellular matrix proteins in influencing or regulating differentiation and remodeling of bone and tissue (Bauer, S, Mark, & Park, 2013).

In the field of dental surgery, some considerable research work has been done to further investigate osseointegration. Du et al established in their work on the ultrastructural relationship between dental implants and osteocytes following osseointegration that there was direct anchorage of osteocytes and dendritic processes to titanium implant surface *in vivo* (Du, Ivanovski, Hamlet, Feng, & Xiao, 2016). It has also been postulated in another study that the *in vivo* performance of the micro arc oxidation coated strontium (MAO-Sr) coated dental implants with regards rapid osseointegration was comparable to that of commercially available titanium implants and so suggesting a novel implant which enhance osseointegration through angiogenesis and osteogenesis (W. Zhang et al., 2016). Despite the above studies, there is no work in the established literature directed at addressing implant failure due to lack of osseointegration in sickle cell disease patients. In addition, to the best of our knowledge there has been no experimental models described in the literature, which investigates the pathogenesis and *in vivo* sequelae of SCD. This review and the subsequent experimental studies could help lay building blocks for potential future research work in these patients.

1.5 *HIF AND ITS ROLE IN OSSEOINTEGRATION*

Oxygen, an indispensable metabolic substrate in various enzymatic reactions *in vivo* including mitochondrial respiration, is a key regulatory signal in tissue development and homeostasis. For example, during embryonic development, cellular differentiation as well as organ growth and final shape are thought to be modulated by oxygen gradients, which, at least in part, rely on the hypoxia-inducible factor (HIF) signaling pathway to mediate their effect (Maes, Carmeliet, & Schipani, 2012). At a molecular level, a HIF complex contains an *alpha* and *beta* subunit, both of which can be selected from several alternatives. They are members of a large family of transcription factors, which contain a basic helix–loop– helix region, and a PAS domain (named for *Per*, *Arnt/HIF- 1β* and *Sim*). HIF *β* subunits are constitutive and are involved in xenobiotic responses. The *α* subunit is regulatory and is unique to the hypoxic response (Maxwell, 2005). The HIF family comprises three subunits: HIF-1alpha, HIF-2alpha and HIF-3alpha. HIF-2alpha and HIF-3 alpha have limited homology with HIF-1alpha, but all three subunits share the conserved Von Hippel–Lindau (*vhl*) - binding domain and are consequently regulated by hypoxia in the same way as HIF-1 alpha (Wiesener et al., 1998).

There is established evidence that HIF1α promotes angiogenesis and osteogenesis by elevating VEGF levels in osteoblasts. Wang and Wan et al showed in their study that mice overexpressing HIF-1α in osteoblasts through selective deletion of the Von Hippel–Lindau gene (*vhl*) expressed high levels of *VEGF* and developed extremely dense, heavily vascularized long bones and they showed a model for the HIF-1α pathway in bone formation. In this model, osteoblasts residing on the nascent bone surface sense reduced oxygen or nutrient levels and upregulate HIF-1α subunits. Elevated HIF1α transactivates target genes such as VEGF, which then stimulate new blood vessel formation and invasion into bone. This process is exponential, with ever-increasing numbers of new blood vessels introducing more osteoblast progenitors, which then mature and function to form more individual bone formation units (Wang et al., 2007). Wan and Shao et al described in their own *in vitro* study that the hypoxic inducible factor-1 alpha (HIF-1) pathway has been identified as a key component in this molecular process. They demonstrated that overexpression of HIF-1α in mature osteoblasts through disruption of the Von Hippel-Lindau protein profoundly increases angiogenesis and osteogenesis (Wan et al., 2010). However, current literature does not point to evidence of a model used in chronic hypoxia to study the role of HIF in osteogenesis and this is a potential research avenue which could be explored.

The decisive role of the implant surface properties on molecular interactions, cellular function and bone regeneration has been demonstrated extensively in *in vitro* research. However, a thorough understanding of the genetic scale of the onset phase of bone regeneration at the implant interface is required prior to the development of strategies to optimize implant osseointegration (Vandamme et al., 2011b).
It was concluded in a study that angiogenic events are crucial for subsequent implant osseointegration. Their finding that the gene expression of angiogenic markers is differently regulated in normal versus compromised bone at 2 days after implantation strengthens this requirement. This event coincides with a negatively affected osteogenic cell response and results in a suspended implant osseointegration in compromised bone. (Vandamme et al., 2011a).

This now calls into question the role of HIF with regards osseointegration. Vandamme et al also noted in their study that HIF1 alpha expression increased in the initial phase of osseointegration and hence VEGF up-regulation. There are studies which have evaluated the role of implants surfaces and their role in osseointegration. Park J et al showed clearly in their study that the Strontium (Sr) containing oxide layer produced by hydrothermal treatment was effective in improving the osseointegration of Titanium (Ti) – 6Aluminium–4V alloy implants by enhancing differentiation of osteoblastic cells (Park et al., 2010). In addition, studies have provided evidence that osteoblast attachment, as well as alkaline phosphatase (ALP) activity and calcium deposition was enhanced by the immobilized VEGF on the polysaccharide-grafted Titanium. Thus, Titanium substrates modified with polysaccharides conjugated with VEGF can promote osteoblast functions and concurrently reduce bacterial adhesion (Hu et al., 2010).

It has also been determined that osseointegration between tissue engineered bone and dental implants was enhanced by HIF-1α (Zou et al., 2012)**.** However, there is no literature evidence in the role of HIF's molecular physiology being used in promoting the prospect of osseointegration in pathologic bony conditions involving chronic hypoxia. There could therefore be a need to study this in detail scientifically via properly directed and designed research.

1.6 *THE ROLE OF HIF IN CHRONIC HYPOXIA AND ITS EFFECTS OSSEOINTEGRATION*

Human mesenchymal cells (hMSCs) are multipotent cells, as they are capable of differentiating along the osteogenic, adipogenic and chondrogenic lineages as previously demonstrated by numerous studies. Potier and Ferrieria et al described in their own study that temporary exposure of mesenchymal stem cells to hypoxia leads to limited stimulation of angiogenic factor secretion and to persistent down-regulation of several osteoblastic markers, which suggests that exposure of MSC transplanted *in vivo* to hypoxia may affect their bone forming potential. They also established in their study that there was a 2-fold up-regulation of vascular endothelial growth factor (VEGF) expression by hMSCs occurs under hypoxic conditions at both mRNA and protein levels. These findings are in agreement with previous reports that hypoxia increases VEGF expression (Potier et al., 2007). However, a limitation in this study was the methodology in creating the hypoxic environment for cell growth - without the use of modern oxygen incubators accurate readings for the partial pressure of oxygen (O_2) at cellular level would be difficult to achieve**.** Diverse responses to hypoxia have been reported for cultured osteoblasts, including increased synthesis of (VEGF), insulin-like growth factor II (IGF-II), and transforming growth factor β1 (TGF β1) (Akeno, Czyzyk-Krzeska, Gross, & Clemens, 2001; Warren et al., 2001). The long-term effects of hypoxia on the function of osteoblasts, the bone forming cells, have received little direct attention (Utting et al., 2006). It was highlighted in the study that exposure of osteoblasts to hypoxia (2% O_2) for the first 6 days of culture followed by 20% O_2 for the final 12 days resulted in a three-fold decrease in bone nodule formation and the reverse led to a two-fold decrease in bone nodule formation. They saw that the amount of bone nodule formation in cultures subjected to early hypoxia added to that measured in cultures subjected to late hypoxia, approached that of cultures held at 20% O_2 continuously, indicating that osteoblasts are able to recover from hypoxic insult. This type of insult mimics that seen in sickle cell disease patients who may eventually develop necrosis (Utting et al., 2006).

It is worth noting that HIF activation causes angiogenesis, which is a prerequisite for osteogenesis and then osseointegration (Wang et al., 2007). New bone forms only in close connection to blood vessels. The mature bone cell does not survive more than 200 μm away from a blood vessel. First, the blood vessel develops and then the bone follows, a process called angiogenetic osteogenesis (Terheyden, Lang, Bierbaum, & Stadlinger, 2012). Bone formation by osteoblasts is critically dependent on oxygen and provide further evidence for

the vital role of the vasculature in maintaining bone health (Utting et al., 2006). With the understanding that osteoblasts can be genetically modified in order to cause the overexpression of HIF 1 alpha (Wan et al., 2010) and then leading to increased osteogenesis, we propose that this concept be used in an experimental *in vitro* research model. This is to study the degree of osseointegration with these modified osteoblasts when exposed to chronic and repeated hypoxic insults as seen *in vivo* with sickle cell disease (SCD) patients with prosthetic implants. With hypoxia being both a cause and sequelae of SCD (Hargrave, Wade, Evans, Hewes, & Kirkham, 2003), there would be important clinical implications from the above proposal in improving osseointegration in these patients. Mimicking the *in vivo* pathologic microcellular environment seen in these patients, the above model proposed could be the first stepping-stone into developing prosthetic hip implants that would help solve the problem of implant loosening and lack of osseointegration characterized in SCD patients with total hip arthroplasty.

1.7 EXPERIMENTAL MODELS FOR HYPOXIC INDUCIBLE FACTOR (HIF) EXPRESSION

It would be important to explore the various models used in the literature in the over expression of HIF. HIF-1α plays a key role in hypoxia response, including glucose metabolism, vascular remodeling, and erythropoiesis. As this protein is expressed by mammalian cells in response to hypoxia, this leads to various angiogenic and osteogenic processes in which different cells lines have been used to express it *in vitro* (Sun & Peng, 2015).

1.7.1 CELL TYPE

Mesenchymal stem cells (MSC), osteoblast-like osteosarcoma cells (MG63 cells), adipocytes and other cells have been used. However, for the purpose of this review as it is related to avascular necrosis in sickle cell bone disease, we have focused on MSCs and osteoblasts. MSCs have shown increased proliferation under hypoxia with over expression of HIF while other studies have revealed the opposite (Holzwarth et al., 2010; Peng, Shu, Lang, & Yu, 2016). The difference in these results may be due to donor specific differences or origin of tissues from which the MSCs have been isolated. It has been well documented that age-related cellular impairment associated with cell proliferation, senescence and stem cell plasticity has been seen based on the various tissues from which the MSCs have been derived from (Sun & Peng, 2015). MG63 cells on the other hand is known to form a monolayer more rapidly and does this easily (Czekanska, 2011; Heremans, Billiau,

Cassiman, Mulier, & de Somer, 1978). In addition to their long culture life, they do have a surface integrin subunit profile, which has been noted as being effective and useful for cell attachment on various materials, therefore making it a well-used cell type for not only culture studies but also studies involving HIF expression (Czekanska, 2011). It is also worth noting that MG63 cells are much cheaper to purchase than MSCs (Sigma-Aldrich, 2019).

1.7.2 MODEL FOR HIF EXPRESSION (*IN VIVO OR IN VITRO*)

Most of the models used for the expression of HIF were mostly *in vitro* but recently more *in vivo* models have been used to investigate HIF in various specific conditions. It is obvious that *in vitro* models may be much easier to set up and can be modified to investigate various conditions (Liu et al., 2019). *In vivo* models go a long way in validating the expression of HIF, as this is much more akin to what occurs in the human body. *In vivo* models also have the advantage of mimicking authentically the pathologic human conditions hence results are more representative. While *in vivo* models capture the complexity of the macro-process in a living system, investigation of the individual steps is challenging and extracting quantitative mechanistic data is usually very difficult. In contrast, *in vitro* models have reduced physiological relevance, capturing only limited aspects of the microenvironment, but allow control of most experimental variables and permit quantitative analysis. With the drive towards precision medicine being on the rise, it is not uncommon to see *in vitro* research models being adapted for patient specific pathologies and therapies as can be seen and could relate to one of the aims of this index research work (Katt, Placone, Wong, Xu, & Searson, 2016).

1.7.3 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT qPCR) VERSUS ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) IN HIF EXPRESSION

Broadly speaking, most of the models used in the analysis of the expression for HIF have used reverse transcriptase quantitative polymerase chain reaction rather than ELISA (Lai et al., 2017; Sun & Peng, 2015) . There are important reasons for this. ELISA is a protein-based method and qPCR is a DNA-based method- involving RNA isolation and extraction, and so there has been well-documented facts on the pros and cons in their use. ELISA is a quicker and easier method of analysis to carry out, with less tissue handling and unlike qPCR, which involves RNA extraction and cDNA production, which entails much more tissue handling and this could lead to increase of experimental errors due to technique. The qPCR

process however is less expensive and more specific (Adam T. Perestam, 2017). It is crucial to note that there have been models where the two methods have been used to express various proteins and not just HIF (Forooghian, Razavi, & Timms, 2007; Heward, Roux, & Lindsay, 2015; Liu et al., 2019; Roux, Heward, Donnelly, Jones, & Lindsay, 2017). The dual use of both methods has been important in the validation of results. With qPCR used to express the target protein, ELISA has been used to validate the level of expression in some studies in the literature (Dijkstra, van Kempen, Nagtegaal, & Bustin, 2014a; Lampert et al., 2016).

1.8 *CONCLUSION*

This review has helped to highlight the problem of implant loosening due to deficient osseointegration seen in sickle cell disease patients. Existing literature is devoid of answers to this problem. Therefore, this review has shed light on the role of the hypoxic inducible factor (HIF) and its importance in improving osteogenesis in physiologically hypoxic conditions. Established literature have in the past put forward various reasons for high implant component loosening in these patients but there has not been any experimental model documented - either *in vivo or in vitro* which investigates this. In addition, the role of HIF and its effects has not been explored as a possible benefit in improving osseointegration in these patients.

In an attempt to help address the gap in knowledge highlighted with regards the problem of component loosening seen in SCD patients, the aim of this study was to takes steps to develop a method that could help reduce component loosening in SCD patients.

The first objective was to establish whether the shape of the proximal femur in these patients could be a factor in femoral component loosening. As part of this aim, it was important to identify the variation in the shape of the proximal femur in SCD patients when compared with those in osteoarthritic patients.

The second objective was to develop an *in vitro* experimental model which may help form the basis for investigating osseointegration in an oxygen deprived bony environment similar to that seen in the marrow of SCD patients- an environment where femoral implantation takes place for total hip replacement. This was done by over expressing HIF. Furthermore, there was the need to add validity to this model, by assessing the cellular metabolic viability of osteoblasts cultured previously and the results could help serve as a positive indicator for transfer of the model to *in vivo* conditions. This could serve as a foundation for improving osseointegration in SCD patients needing THA as treatment for avascular necrosis of the hip. The study therefore acts as the building block needed for improved implant design tailored to reduce the surgical re-operating burden due to implant loosening in sickle patients and invariably improve their quality of life.

CHAPTER TWO

RADIOGRAPHIC EVALUATION OF THE PROXIMAL FEMUR IN SICKLE CELL DISEASE PATIENTS

2.0 INTRODUCTION

Avascular necrosis (AVN) is the death of bone that results in the collapse of the architectural bony structure, leading to joint pain, bone destruction, and loss of function (Ramos-Casals et al., 2002). Several underlying causes of AVN has been described. In some such as, proximal femur fracture, sickle cell anaemia, or decompression illness, the cause seems evident, while in others, such as treatment with glucocorticoids (GC) or alcohol consumption, the cause is not so clear. The common denominator is the femoral head liability to ischemia, but the pathogenic mechanism is variable. In the case of a femoral fracture, compression or rupture of a blood vessel is the cause of osteonecrosis (ON), while in sickle cell anaemia and in decompression illness, it is attributed to alterations in sinusoidal circulation (Martinez-Ferrer, Peris, & Guanabens, 2007).

AVN of the femoral head develops in 3-19% of patients with sickle cell disease and frequently this is bilateral. Sickle cell osteonecrosis is a specific entity characterised microscopically by diffused necrotic lesions of bone trabeculae and marrow, associated with an inflammatory process and new bone formation. This new apposition is responsible for osteosclerosis leading to a homogeneous increase of density of the head (Mukisi-Mukaza, Gomez-Brouchet, Donkerwolcke, Hinsenkamp, & Burny, 2011). Intravascular sickling causes thrombosis and then ischemia, resulting in infarcts in the femoral head, which progress to avascular necrosis. Chughtai et al noted in their systematic and instructional review that a definitive pathogenic mechanism for the osteonecrosis remains elusive (Chughtai et al., 2017).

Total hip replacement (THR) is therefore, indicated in such patients. It has also been seen that hip resurfacing arthroplasty could be successful in patients with osteonecrosis of the femoral head. However, various studies have shown high complication rates in these groups of patients who had either cemented and uncemented implants (Amstutz & Le Duff, 2016). Hickman et al in his study reported a 20% early revision rate in the patients who had THR. This was mainly due to femoral osteolysis and polythene acetabular wear (Hickman &

Lachiewicz, 1997). In addition, Bishop et al in his cohort group of sickle cell disease patients who underwent THR had a 15% revision rate mainly due to deep infection and implant loosening. Some theories have been put forward as reasons for this pattern, which include abnormal immune system, functional asplenia, and poor blood supply to bone. The reasons for this higher failure rate of the femoral component in many previous studies is mostly unknown (Tingart et al., 2009). Reported outcomes of total hip replacement in the AVN population have not been as good as in patients with osteoarthritis. Patients with osteonecrosis tend to be younger and more active than OA patients, which may account for some of the discrepancy (Brinker, Rosenberg, Kull, & Cox, 1996; Cornell & Ranawat, 1986; Ortiguera et al., 1999; Saito, Saito, Nishina, Ohzono, & Ono, 1989). Alternatively, changes in the bone quality in the proximal femur seen in osteonecrosis may compromise the result (Radl, Egner, Hungerford, Rehak, & Windhager, 2005; Radl, Hungerford, Materna, Rehak, & Windhager, 2005). Studies in normal patients undergoing primary hip replacements have shown good implant osseointegration. The average age of patients with osteonecrosis of the hip is 38 years. However, this patient group is at higher risk of implant failure, which may be due, at least in part, to their youth and activity (Lennon & Prendergast, 2001). Kim Y.H. et al have suggested that maintaining the mechanical integrity of the cement mantle could mean reducing the peak stress on the cement/bone and cement/prosthesis interfaces and this reduces loosening rates. They also noted in their study that there was no significant difference in failure rates between cemented and uncemented stems in patients with AVN (Y. H. Kim et al., 2011). It has been noted in a previous literature systematic review that there was a significant higher arthroplasty revision rate in patients with AVN secondary to sickle cell disease as compared with those with AVN from other causes (Johannson et al., 2011).

In a large series of consecutive three hundred and twelve (312) patients, aseptic loosening of the acetabular component occurred in 25 (8%) of the 312 hips after an average follow up of 13 years. Aseptic loosening of the femoral component was as common as that of the acetabular component. The incidence of aseptic loosening in this study with patients who had sickle cell disease is in contrast with the very high rate of loosening of 10% to 38% that has been reported in previous series with a very small number of patients and a short follow up (Hernigou et al., 2008). The author of this study attributed the reduction in the loosening rate to proximal canal filling prosthesis. Furthermore, it can be argued that the recent studies have shown improved revision rates and encouraging results with the use of uncemented implants in SCD patients. Better-designed prosthesis based on the shape of the proximal femur could be responsible for this.

There is a scientific rationale and some evidence supporting the hypothesis that the shape of the proximal femur is associated with compartment-specific knee OA and also it appears to influence the pattern of acetabular wear occurring over time (Streit, Levine, Barrett, Cooperman, & Goldberg, 2013; Wise et al., 2014). It has also been observed that the shape of the proximal femur is an important factor in understanding the relationship between hip joint mechanics, gait pattern and the development of hip OA (Pedoia et al., 2018). However, there is no documented structural reason in the shape of the proximal femur in sickle cell disease patients which may cause implant loosening and hence the reason for this study in investigating this. It is also important to note that there is no documented pattern for the shape of proximal femur in these group of patients hence the need to explore this through this study.

The aim of this study was to assess the variation in the proximal femoral canal anatomy in sickle cell disease patients and compare with similar cohort of osteoarthritic patients. Furthermore, the study sought to deduce if the shape of the proximal femur could have any bearing on the femoral component loosening rate in this group of patients which no other study to the best of our knowledge has established.

2.1 PATIENTS AND METHODS

The standardized anterior posterior radiographs of forty-two (42) sickle cell disease patients with degenerative hip osteoarthritis secondary to avascular necrosis and another matched cohort of forty-two (42) patients with primary degenerative hip osteoarthritis (OA) were initially taken and evaluated on a standard imaging software. Patients x-rays were taken as part of their routine clinical assessment for diagnosis. The study had local Research and Development approval and informed consent was taken from patients for their radiographs to be used. Both groups of patients sampled were from the indigenous local population in Ghana, West Africa who had presented to two local orthopaedic hospitals. The two sample cohorts were matched for age and gender.

2.1.1 RADIOGRAPHIC MEASUREMENT PROTOCOL

The radiographic measurements were taken using the anterior-posterior (AP) views of plain radiographs using a standardized X-ray imaging software, Osiri X Lite (Copyright © 2016 Pixmeo). The lateral views were not available during the evaluation. Indices measured were

canal-to-calcar ratio (CC ratio) and the cortical index (CI) (Dorr et al., 1993b) (Figure 2.1). The CC ratio and CI (anteroposterior views) were measured using the method described by Dorr et al and subsequently used by other clinical researchers who have undertaken studies on the proximal femoral geometry. CC ratio was determined using measurements from two points on anteroposterior plain radiographs of the hip. These two points were established on the medial and lateral aspects of the medullary cavity 10 cm distal from the lesser trochanter and another two points are established on the medial and lateral aspects of the medullary cavity 3 cm distal from the center of the lesser trochanter. The CC ratio was obtained by dividing the distance **X** of the medullary cavity at the point 10 cm distal from the center of the lesser trochanter by the distance **Y** at the point at the center of the lesser trochanter. This is where the two lines formed by the two points on the internal aspect and the two points on the lateral aspect of the medullary cavity pass- **X/Y** *(Figure 2.1).* CI was determined from measurements on anteroposterior of the hip. This was obtained by dividing the thickness of the femoral bone cortex at the point 10 cm distal from the lesser trochanter (distance of the lateral aspect of the femoral bone cortex Z minus distance of the medullary cavity X) by the distance of the lateral aspect of the femoral bone cortex Z (i.e. **CI= Z-X/Z**). The measurements as noted-proximal femoral canal (Y), mid-diaphysis canal(X), extra medullary canal (Z) diameters- were made in centimeters (cm). The calcar-canal ratio (CC): X/Y and the cortical index (CI): Z-X/Z was calculated from the above measurements and the mean determined from both groups of patients (Al-Nasiry, Geusens, Hanssens, Luyten, & Pijnenborg, 2007; Dorr et al., 1993a; Merle, 2014; Nakaya, Takao, Hamada, Sakai, & Sugano, 2018). Based on the indices determined, the patients' proximal femur shape was classified into three groups – A, B, C according to Dorr's classification with the values of CI and CC as stated in Table 2.1 (Dorr et al., 1993a; Husmann, Rubin, Leyvraz, de Roguin, & Argenson, 1997; Nakaya et al., 2018). According to the Dorr's description of the proximal femoral morphology- *(Figure 2.2)*, Type A exhibited thick cortices that begin at the distal end of the lesser trochanter and thicken quickly, producing a funnel shape and a narrow diaphyseal canal. Type B exhibited bone loss proximally and widening of the diaphyseal canal. Type C exhibited considerable loss of the thickness of the cortices resulting in a wide intramedullary canal and a fuzzy appearance to the bone cortices, which can be described as stove-shaped (Nash & Harris, 2014).

2.1.2 OBSERVER RELIABILITY

In order to ensure reliability of the radiographic measurements, three observers - orthopaedic registrar, general practitioner (GP), and medical science student were asked to take the above radiographic measurements independently as shown in *Figure 2.1*. Copies of this document showing the measurement protocol was made available to them for reference and the protocol was explained. The level of agreement using the intra-class coefficient (ICC) was determined for both intra-observer and inter-observer measurements. Based on the 95% confident interval of the ICC estimate, values less than 0.5, between 0.5 and 0.75, between 0.75 and 0.9, and greater than 0.90 are indicative of poor, moderate, good, and excellent reliability respectively (Koo & Li, 2016; Merle, 2014).The intra-observer measurements were taken with observers taking measurements on two separate occasions at least one month apart.

STATISTICAL ANALYSIS

Statistical analysis was carried out using the SPSS statistical software programme (SPSS Inc. IBM version 22). There were no previous similar comparative studies in the literature and so there was no representative sample sizes to compare with. However, in order to be pragmatic with the study, a post hoc power analysis was done bearing in mind this could be seen as an initial exploratory observational study. Power calculations for the sample sizes was evaluated using G*power®2017: version 3.1.9.2 free to use statistical software. The power analysis for the SC patient sample sizes revealed that on the basis of the mean, the effect size observed in the study (d=1.05) and with n=42 would be needed to obtain statistical power of 0.99 level. Also, for the OA patient group based on the mean, the effect size (d=0.69) and with n=42 would be needed to obtain a statistical power of 0.88.

Parametric tests were used to assess the normality of the data and the Welch independent sample t test was used to evaluate the level of significance for the cortical indices and calcar-canal ratios between both groups of patients. Significance level was $p<0.05$ unless otherwise indicated. Pearson correlations was used to assess linear comparable variables while Spearman's rank correlation was used to evaluate the relationship between non-linear variables. One-way ANOVA test was used for differences in mean within and between groups.

A Welch t -test was run to determine if there were differences in the calcar canal ratio (CC) and the cortical index (CI) between sickle cell and osteoarthritic patients due to the assumption of homogeneity of variance being violated as assessed by Levene's test for

equality of variances (p=0.000037, p value <0.05).(Statistics, 2015a). There were no outliers in the data as assessed by inspection of the box plots (*Figures A10 and A11).* The CC and CI data for both patient groups were normally distributed as assessed by the visual inspection of the N-N Q-plots. (*Figures A12-A15)*. The one-way ANOVA was run to determine if the CI in both groups of patients was different for the various Dorr's femur types. Both groups of data were normally distributed.

The Spearman's rank order correlation was used to assess the relationship between the various parameters CI, CC ratio and BMI in the sickle cell group (Statistics, 2018b). Preliminary analysis showed the relationship to be non-monotonic as assess by the visual inspection of the scatter plot *(Figure A16, A17).* When analysing the correlation within the OA group, Pearson's correlation was used as the only exception to Spearman's in order to assess the relationship between age and CC ratio. The preliminary assessment showed a linear relationship between both variables with data showing a normal distribution as confirmed by the scatter plot and Q-Q plot. (Statistics, 2018a) (*Figures A18, A19)*.

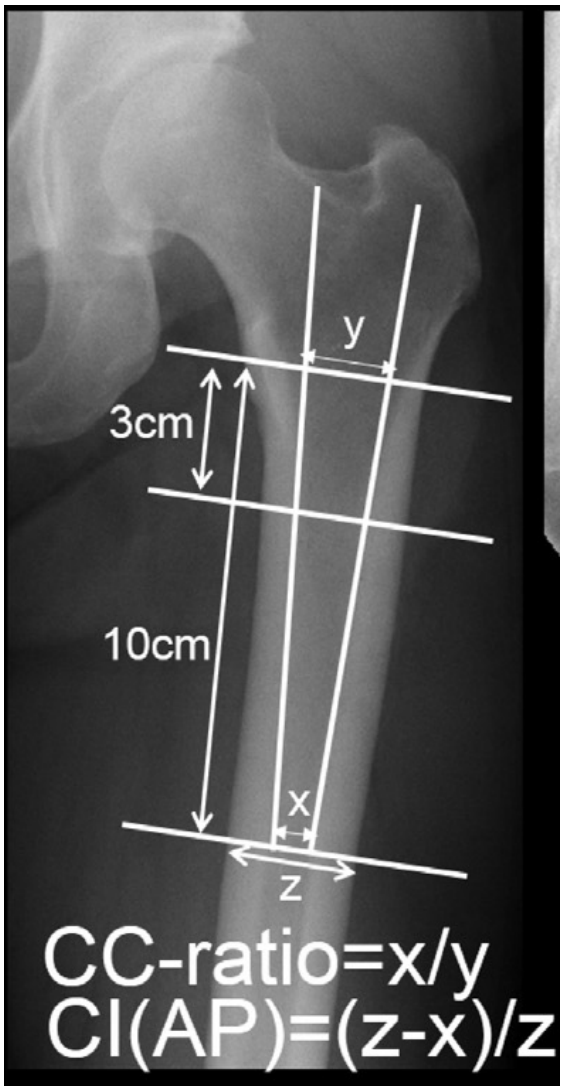

Figure 2.1*: Plain radiography showing descriptive protocol measuring each parameter is shown. CC ratio: canal-to-calcar ratio; CI: cortical index*

Reference- Nakaya R, et al. 2018.

Table 2.1- *Showing Dorr's classification of Femur Shape types based of values of Calcar Canal ratio and cortical index. Reference - (Dorr et al., 1993a).*

	A	B	C
Calcar Canal- CC Ratio	< 0.57	0.59 – 0.61	> 0.62
Cortical Index- CI (Anterior-Posterior view)	> 0.58	0.43 – 0.57	< 0.42

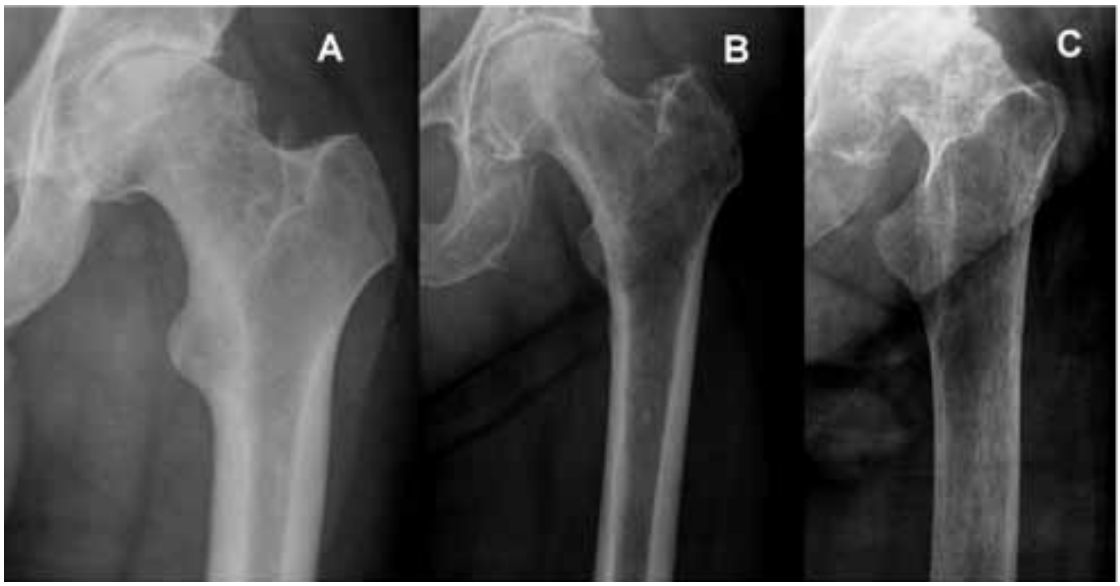

FIGURE 2.2 *Showing Dorr Types A, B, C Proximal femur morphology*

Reference-(Nash & Harris, 2014). Image reproduced with permission

2.2 RESULTS

2.2.1: COMPARISON BETWEEN SICKLE CELL AND OSTEOARTHRITIC PATIENTS

In the sickle cell cohort of patients evaluated, there were 29 females and 13 males. The mean age was 41.5 years (range 23-68) and the body mass index (BMI) of these patients was 26.9 (range 18.5-45.9). (*Table 2.2)* The mean cortical index (CI) was evaluated as 0.49(0.38-0.83), while the mean calcar to canal (CC) ratio was 0.47(0.27-0.89). *(Table 2.3)*

For the osteoarthritic group of patients evaluated, there were 29 females and 13 males. The mean age was 49.5yrs (range 23-76) and average body mass index was 28.5 (range 20-60.2). (*Table 2.2).* The mean cortical index (CI) was 0.64 (0.20-0.90) and calcar canal ratio (CC) was 0.36 (0.10-0.81). *(Table 2.3)*

Table 2.2 *Showing the demographic parameters for both patient groups*

SICKLE CELL PATIENTS			
		Range Interval	**Standard Deviation (SD)**
Male (Number)	19		
Female (Number)	23		
Age (Mean)	41.5 years	23 - 68	10.73
Body Mass index (BMI)	26.9	18.5 - 45.9	6.71
OSTEOARTHRITIC PATIENTS			
Male (Number)	19		
Female (Number)	23		
Age (Mean)	41.5 years	23 - 76	10.73
Body Mass index (BMI)	28.5	20.0 - 60.2	8.04

Table 2.3 *Showing Comparison of radiographic parameters between both patient groups*

	Sickle Cell patients	**Osteoarthritic patients**	**Significance (p value <0.05)**
Dorr Type (Numbers)			
A	34	34	
B	4	5	
C	4	3	
Calcar canal ratio (CC)	0.47	0.36	0.002*
Range Interval	0.31- 0.66	0.1- 0.81	
Standard Deviation (SD)	0.09	0.20	
Cortical index (CI)	0.49	0.64	0.00037*
Range Interval	0.37- 0.62	0.20 - 0.90	
Standard Deviation (SD)	6.71	0.20	

**Correlation is significant at the 0.05 level (2-tailed)*

The cortical index in the SC patients mean (M) = 0.49, SD =0.09 was lower than that of OA patients mean (M) = 0.64, SD = 0.20, this was statistically significant p=0.00037, (p value < 0.05) *(Figure 2.3).*

The calcar canal ratio in sickle cell patients was significantly higher mean (M) = 0.47, standard deviation (SD) = 0.08 than that of osteoarthritic patients mean (M) = 0.36, standard deviation (SD) = 0.2, and this was statistically significant, p=0.002, (p value <0.05) *(Figure 2.4).*

There was no statistically significant difference in the cortical index (CI) and calcar canal ratio (CC) between males and females in the SC group. (p=0.845 for CC: p value>0.05 and p=0.135 for CI: p value >0.05) (*Table 2.3, Figure 2.3).* With regards the OA patients, there was a difference in the CI for males, mean (M) =0.53, SD=0.22 compared with that of females M=0.71, SD=0.14. *(Figure 2.4)*. There was a statistically significant difference in the mean (m) = -0.18, S.E=0.07, t (16.7) = -2.71, p = 0.015 (p value <0.05) (*Table 2.4)* (Statistics, 2015a).

For SCD patients there was no statistical difference in CI and the different femur types, Welch's F (2, 4.512) =1.07, p= 0.416 (p=0.05) (Statistics, 2017a). This was the same for the OA group as well. There was no statistical difference in CI and the different femur types, Welch's F (2, 4.66) =3.07, p= 0.14 (p value <0.05) *(Table 2.5)* (Statistics, 2017a).

Table 2.4 *Showing Comparison of radiographic parameters between both patient groups based on gender*

Sickle cell patients			
	Male	**Female**	**Significance-2 tailed (p<0.05)**
Cortical Index (CI)	0.51	0.48	0.135
Standard Deviation (SD)	0.05	0.06	
Calcar Canal Ratio (CC)	0.46	0.47	0.845
Standard Deviation (SD)	0.07	0.09	
Osteoarthritic Patients			
Cortical Index (CI)	0.53	0.71	0.015*
Standard Deviation (SD)	0.22	0.14	
Calcar Canal Ratio (CC)	0.47	0.30	0.015*
Standard Deviation (SD)	0.22	0.13	

**Correlation is significant at the 0.05 level (2-tailed)*

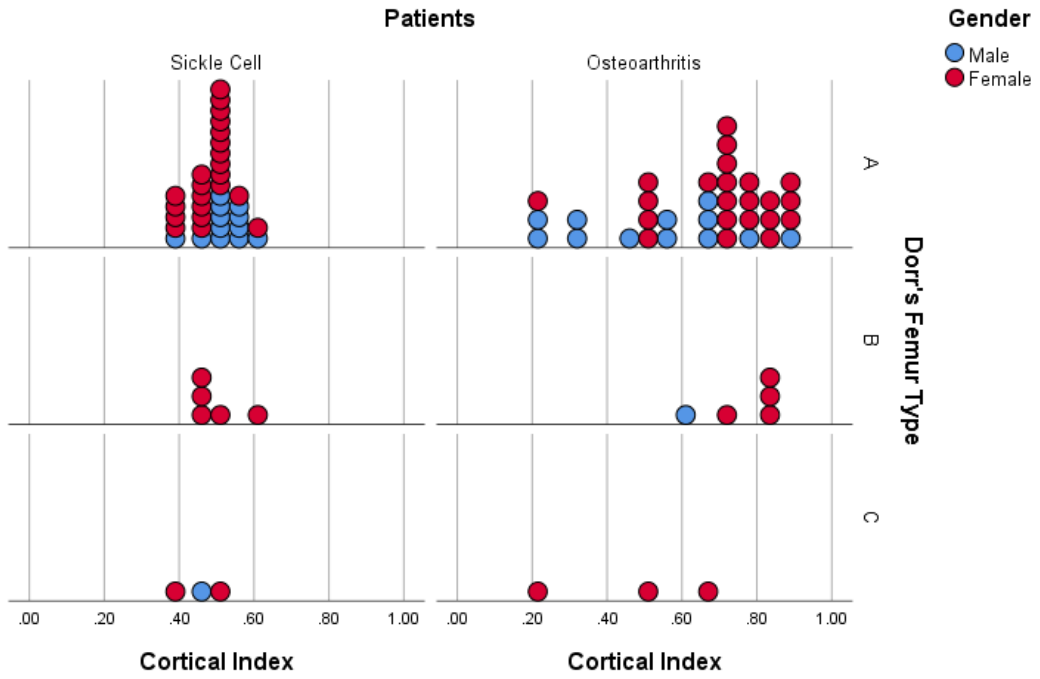

Figure 2.3 *Shows simple dot plot of cortical index by gender and patient types (sickle cell/osteoarthritis) based on Dorr's femur type. It is noted that there is more CI variability in the OA group. There was no statistically significant difference in the cortical index between males and females in the sickle cell group, p value >0.05; however, there was significant difference in the CI between genders for the osteoarthritic group, p value < 0.05.*

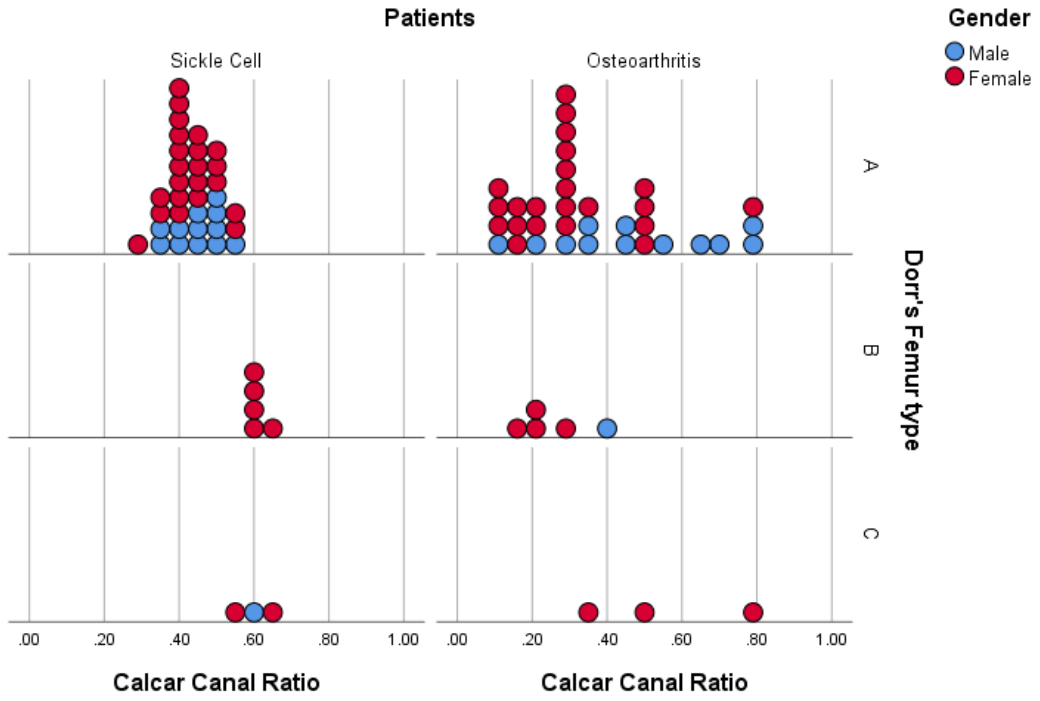

Figure 2.4 *Shows simple dot plot of Calcar canal ratio by gender and patient types (sickle cell/osteoarthritis) based on Dorr's femur type. It is noted that there is more CC ratio variability in the OA group There was no significant statistical difference in the calcar canal ratio between males and females in the sickle cell group p value >0.05; however, there was significant difference in the CC ratio between genders for the osteoarthritic group, p value < 0.05.*

Table 2.5 *Showing the Dorr's femur types with corresponding cortical index (CI)*

SICKLE CELL PATIENTS	NUMBER	CORTICAL INDEX- (MEAN)	STANDARD DEVIATION (SD)
Dorr femur type			
A	34	0.50	0.06
B	5	0.49	0.62
C	3	0.45	0.05
Significance (p value <0.05) p =0.41			
OSTEOARTHRITIC PATIENTS			
Dorr femur type			
A	34	0.64	0.20
B	4	0.76	0.10
C	4	0.47	0.22
Significance (p value <0.05) p = 0.14			

2.2c CORRELATIONS

For the sickle cell disease patients, there was no statistically significant correlation between the CC ratio and BMI, r (40) = - 0.159, p = 0.315 (p>0.05), two tailed (*Table 2.6).* It was also noted that there was no statistically significant relationship between CI and BMI, r (40) = 0.048, p=0.776 (p>0.05), two tailed. However, there was significant correlation between age and the BMI of SC patients, r (40) = 0.35, p=0.023 (p<0.05), two tailed *(Table 2.6).*

With regards the correlations for the OA group, there was a statistically significant positive correlation between age and CC, r (40) = +0.518, p=0.0004 (p<0.01), two tailed. (*Table 2.7*) (Statistics, 2018b). There was also a statistically significant negative relationship between CI and age, r (40) = - 0.51, p= 0.001 (p<0.01), two tailed. It was also seen that there was a statistically significant correlation between BMI and age, r (40) = 0.352, p=0.022 (p<0.05), two tailed. (*Table 2.8*)

Table 2.6 *Showing Spearman's correlation between Age and BMI with radiologic parameters deduced in Sickle Cell group.*

	Correlation (rho)	**Significance (2-tailed)**
Age vs Calcar Canal Ratio (CC)	0.021	0.896
Age vs Cortical Index (CI)	0.038	0.809
Age vs Body Mass Index (BMI)	0.35	0.023*
Body Mass Index vs Calcar canal ratio	-0.159	0.315
Body Mass Index vs Cortical Index	0.046	0.776

**Correlation is significant at the 0.05 level (2-tailed)*

Table 2.7 *Showing Pearson's correlation between Age and Calcar Canal ratio in Osteoarthritic patient group*

	Correlation (rho)	Significance (2-tailed)
Age versus Calcar canal ratio	0.528	0.0004**

***Correlation is significant at 0.01 level (2-tailed)*

Table 2.8 *Showing Spearman's correlation between Age and BMI with radiologic parameters deduced in Osteoarthritic patient group.*

	Correlation (rho)	Significance (2-tailed)
Age vs Cortical Index (CI)	0.51	0.001**
Age vs Body Mass Index (BMI)	0.35	0.022*
Body Mass Index vs Calcar Canal ratio	0.127	0.423
Body Mass Index vs Cortical Index	-0.102	0.522

*** Correlation is significant at the 0.01 level (2-tailed)*

** Correlation is significant at the 0.05 level (2-tailed)*

2.2.1 LEVEL OF AGREEMENT AMONGST OBSERVERS

INTRAOBSERVER RELIABILITY

The intra-observer Intra-class correlation coefficient (ICC) was found to be excellent among all three observers across all the radiographic measurements in both cohorts of patients ranged from 0.89 to 0.99 (p value <0.001).

INTEROBSERVER RELIABILITY

The inter-observer reliability data showed excellent agreement amongst observers in all radiographic measurements in the sickle cell cohort, range 0.91 to 0.99, p value <0.001 and there was moderate to excellent agreement amongst observers in the osteoarthritic cohort in all radiographic measurements taken, range 0.47 to 0.99 p value<0.001. Please see *Tables A1-A6*

2.3 DISCUSSION

This radiographic study had the objective of confirming or refuting the hypothesis that the variation in the shape of the proximal femur maybe responsible for femoral implant loosening in sickle cell disease patients with total hip arthroplasty. Clark et al noted in their cadaveric study that the normal shape of the proximal femur is variable, and variations in the absence of intrinsic bone disease appear to reflect adaptation to physiologic variations in the line of action of muscle forces (Clark, Freeman, & Witham, 1987). In view of this, Bahari et al in their study attempted the prediction of shape and internal structure of the proximal femur using a modified level set method for structural topology optimisation (Bahari, Farahmand, Rouhi, & Movahhedy, 2012). It could be said that these variations in the shape of the proximal femur can be seen in various pathologic conditions but established literature has little of this information. Hammer et al determined that the forces acting through these trabeculae on the proximal femur can affect its overall shape and configuration in its coronal plane (Hammer, 2002). However, this study did not present any evidence concerning the relationship between variations in femur shape and pathology. There are studies however that observed these structural variations in the proximal femur and have proposed that this helps in assessing the risk of osteoporosis and fragility fractures (Gregory et al., 2004; Lindner et al., 2015). The results from this study though not an autopsy nor finite modelling assessment like previous studies cited above, suggests a similar pathologic risk. It was deduced from this work that most of the patients in the SCD cohort had Dorr's type A

proximal femur funnel shape but less so when compared with OA patients *(Table 2.3)*. It was also noted that there was lower cortical index than that seen in OA patients meaning thinner cortices in sickle cell patients. The combination of these two findings could suggest an increased risk of periprosthetic fractures and loosening with the use of uncemented implants as documented in recent studies (Ilyas et al., 2018). There is no evidence in documented literature that objectively correlates the risk of implant loosening in SCD patients who have had THR with the shape of the proximal femur. However, there could be a case in our findings with OA patients having more funnel shaped femurs allowing for more press fitting femoral implant fixation with the use of uncemented prosthesis. The outcome of the largest series of SCD with cemented THR showed very promising results with low failure rate of the femoral component compared to other series in the literature. The authors ascribed this to the "French Paradox Technique" - where rectangular medullary canal filling femoral prosthesis (without obtaining continuous cement mantle) was used allowing for direct load transfer to bone by close cortical contact (Hernigou et al., 2008). It could therefore be postulated that this was possible due to the geometry of the proximal femur of these SCD patients as seen in this study. The less funnel shaped femurs seen here when compared to OA patients *(Table 2.3)* could be a contributing factor to allowing this technique to be used in the large series earlier described and hence the low implant failure rate.

Some studies have been able to support the relationship between the development of disease pattern of hip osteoarthritis and the shape of the proximal femur (Lindner et al., 2015; Radl, Egner, et al., 2005). However, the question on whether the shape of the proximal femur has a causative role in the development of implant loosening still needs answers and this study sheds some light on this. In this cohort of patients who are all black Africans, it was observed that the average cortical index was marginally higher indicating higher cortical thickness, bone strength and increased trabecular bone mineral density. This finding is similar to conclusions noted by Marshall L.M. et al in their study of the race and ethnic variation in proximal femur structure (Marshall et al., 2008). The afore mentioned parameters should negate implant loosening but this may not be the case in SCD patients with THR. The evaluation deduced in this study noted that there was no significant difference in the cortical indices and the types of femur shapes in both groups of patients *(Table 2.5).* However, this is in contrast to previous radiologic studies (Jennings, Thiele, & Krynetskiy, 2016; Nakaya et al., 2018). It is also worth noting that because of the lack of clear difference in the CI of the various types of femur shapes in the evaluation of the SC patients, it could be difficult to predict the risk of periprosthetic fractures and so invariably leading to implant loosening. This is in contrast to other studies relating cortical thickness to this risk (Nash & Harris, 2014). It is also important to note from this study that there was no relationship

between age and CI, CC in the sickle cell group *(Table 2.6).* This finding is also in contrast with other findings in the literature (Dorr et al., 1993a; Dorr, Wan, Song, & Ranawat, 1998; Tsubosaka et al., 2018). However, in the OA group with the increase in age, there was increase in CC *(Table 2.7)* meaning that older patients had less funnel shaped femurs. Furthermore, it was confirmed that with the increase in age in the OA group, there was decrease in the cortical thickness. These correlation findings were the opposite in the sickle cell disease group and so it would be accurate to suggest that SCD patients' femur shape may not follow established known physiologic patterns.

Dorr et al observed in his study that patients with high cortical index and lower calcar canal ratio have funnel shaped proximal femurs which is suitable for press fit implant fixation (Dorr et al., 1993b). The assessment deduced from this study of the radiographs of SCD patients establishes this finding as well but it still leaves questions about the reason for high implant failure in these patients unanswered. The mean age of patients in this study is similar to previous studies, which show a younger age group, and various observers have highlighted the level of activity as a major cause of implant failure in this group of patients (Dudkiewicz et al., 2004; Hernigou et al., 1993). However, Radl et al in their study involving a similar number in his cohort of patients as ours, showed that patients with total hip arthroplasty after AVN of the femoral head associated with a systemic disease (steroid medication, alcohol abuse and sickle cell disease) are at higher risk of implant failure compared with patients with idiopathic or post-traumatic osteonecrosis. They suggested that the higher failure rates after total hip arthroplasty in patients with osteonecrosis of the femoral head associated with a systemic disease might be related to abnormal bone quality. This is probably accelerated by the higher mechanical demand in this young patient population (Radl, Egner, et al., 2005). The results of our study support this view, with lower CI and less funnel shaped femurs in our sickle cell group. However, recent series have confirmed that there has been no difference in implant survival and failure rate between patients who had THR with AVN from SCD and those who had the same procedure due to osteonecrosis from other causes (Issa et al., 2013). There is however no objective scientific study in SCD patients proving this hypothesis. This study sheds light on the fact that the shape of the proximal femur in these patients is ideal for implant fixation and does not answer the prevailing question of why the high rate of implant loosening which may be from lack of osseointegration. However, it was also noted in this study that the SCD cohort of patients had a higher cortical index and lower calcar canal ratio as compared with other published cohorts of Caucasian osteoarthritic patients in the literature (Dorr et al., 1993b; Marshall et al., 2008). When comparing the cortical index and calcar canal ratio in the SCD group with that in the OA group in this study, it was noted that there was a slight discrepancy with significantly higher CC ratio in the SC

group meaning more funnel shaped proximal femur in the OA group. Furthermore, this study may suggest that African patients have more funnel shaped femurs as compared to Caucasians studies in the literature.

To the best of our knowledge there are no other previous studies looking at this radiologic comparison between SCD proximal femur and that of OA patients. This can be seen as the strength for this work but we are aware that this study has limitations. The low sample size may only allow for significant postulations to be made and not definitive conclusions. Our study only had forty-two patients compared to other studies with greater sample sizes (Dorr et al., 1993a; Jennings et al., 2016; Nakaya et al., 2018; Nash & Harris, 2014; Tsubosaka et al., 2018). The measurements from which the cortical indices were calculated, was from anterior posterior plain x-rays views only as the lateral views were not available for the study due to health care expenses as patients still largely pay for healthcare cost in the host sample country (A. George & Ofori-Atta, 2019; A. O. George & Ofori-Atta, 2009). This did not allow for lateral view verification of the cortical index in these patients. Measurements were also taken on non-digitalized x-ray views, which could impair on the accuracy of measurements *(Figure A1).* The use of CT scan images would have conferred more accuracy to the measurements taken (Hoenecke, Hermida, Flores-Hernandez, & D'Lima, 2010; Wines & McNicol, 2006). Furthermore there were no objective outcome measures in the form of post-operative radiographs which allows for femoral stem fixation assessment for loosening using the Charnley and Gruen methods for cemented (Gruen, McNeice, & Amstutz, 1979) and the Engh method for uncemented prostheses (Engh, Massin, & Suthers, 1990). Although there were three observers for the radiographic measurements' reliability validation, one of the observers was a university medical student, the others were a general practitioner and another an orthopaedic registrar. The observer variability could have an impact on the measurements taken from the plain x-rays. (Hanley et al., 2017) *(Tables A4-A6)*.

It is clear that these SCD patients do have funnel shaped proximal femurs and hence implant sizing maybe an issue as experienced in these patients and further causing intraoperative periprosthetic fractures. Custom designed implants are already being used in hip arthroplasty of patients with congenital dysplasia of the hip (Koulouvaris, Stafylas, & Xenakis, 2008). As the standard of care and life expectancy of sickle cell disease patients improves, the need for optimal results in those needing hip arthroplasty is crucial. This study highlights the different femoral shape geometry in these patients, and so appropriate implant use and design for them cannot be overlooked.

2.4 CONCLUSION

According to Dorr classification of types of variations in proximal femoral anatomy, x-ray measurements showing higher calcar to canal ratios, suggesting less funnel shaped proximal femurs in sickle cell disease patients compared with non-sickle cell OA patients. This is clearly seen in the above measurements determined in this sample of sickle cell disease patients. However, when compared to OA patients, SCD patients had significantly thinner cortices. It is therefore suggested that this could influence femoral stem fixation and increase risk of periprosthetic fractures leading to more stem failures. The results therefore imply that the structural anatomy of these patients' proximal femurs could contribute to implant loosening. It can be concluded that sickle cell disease bone does not follow the normal physiologic trend of decreasing cortical thickness with age and this would be useful to surgeons when carrying out hip replacements in these patients. This study though with its limitations, highlights that the funnel shaped proximal femur with diminished cortical thickness seen in these group of patients makes the case for needed intra operative precautions in sickle cell patients with AVN having hip arthroplasty. Properly designed prospective studies of SCD patients with THR from AVN together with *in vivo* and *in vitro* research models could help highlight other factors that could answer the question of osseointegration in these patients.

CHAPTER THREE

EXPERIMENTAL STUDY TO INVESTIGATE A POTENTIAL MODEL FOR IMPROVED OSSEOINTEGRATION IN SICKLE CELL BONE DISEASE PATIENTS WITH AVASCULAR NECROSIS

3.0 INTRODUCTION

Having established in the previous chapter that the structural anatomy of the proximal femur in sickle cell disease patients may have some basis in the cause of femoral stem prosthesis loosening, then there could also be some truth in the lack of implant osseointegration in these patients which could be another contributing factor to femoral stem loosening. It would be important to establish a model to help investigate the enhancement of this physiologic phenomenon.

Sickle cell disease (SCD) is an inherited blood disorder characterized by adherent, rigid, abnormally shaped erythrocytes that occlude blood vessels and compromise blood and oxygen supply to tissues and organs. ‘‘Hypoxemia,’’ which is due to decreased arterial blood oxygen content, decreased oxygen carrying capacity due to chronic anaemia, and oxyhaemoglobin desaturation, is a well-documented phenomenon in patients with SCD (Caboot & Allen, 2014). Studies have shown that hypoxia in these patients leads to higher incidence of vasocclusive crisis (Setty, Stuart, Dampier, Brodecki, & Allen, 2003). Although vasocculsion can be seen in any organ in the body, it is common in the bone marrow because of marrow hyper cellularity leading to impaired blood flow and regional hypoxia, which is chronic (Almeida & Roberts, 2005). This cascade of events causes avascular necrosis which is the bony environment in which hip femoral prosthesis are implanted in sickle cell disease patients. In a study where longitudinal prospective controlled evaluation of magnetic resonance imaging (MRI) of long bones in sickle cell patients was done, it was concluded that the increased severity of patchiness of the long-bone MRI in older patients with SCD was a result of recurrent sickling with progressive, perivascular fibrosis in the marrow (Mankad et al., 1990).

Despite the above, the role of the hypoxic inducible factor (HIF) in these patients has been called into question. Recent studies show that HIF and Hypoxic mimicking agents (HMA) trigger the initiation and promotion of angiogenic-osteogenic cascade events. *In vitro* and animal studies involving genetic manipulation of individual components of the HIFs and HMAs have provided clues to how angiogenic-osteogenic coupling is achieved. This foundational work is important in understanding the therapeutic basis of bone regeneration and repair (Mamalis & Cochran, 2011). However, there has been paucity of studies investigating how HIF could be over expressed under chronic hypoxic conditions akin to that seen in sickle cell disease patients to help form a template for tackling the matter of macro cellular avascular necrosis. Angiogenesis and osteogenesis are tightly coupled during bone development and regeneration, and the hypoxia-inducible factor-1 alpha (HIF-1) pathway has been identified as a key component in this process studies have shown (Wan et al., 2010). There are still no established experimental models showing how this knowledge can be used for the evaluation of bone implant integration and hence suggest ways of improving osseointegration in sickle cell disease patients with hip arthroplasty, thereby preventing increased implant loosening.

It is fair to say some experimental models have looked at osseointegration in which the concept of hypoxia has been modulated in relation to this phenomenon (Maes et al., 2012), but no *in vitro* models in the literature have be able to mimic similar hypoxic environment in pathologic bone conditions such as SCD. Establishing such *in vivo* models in animals can be difficult because SCD is also a genetic abnormality whose sequelae is chronic bony hypoxia and this can be an uphill task mimicking this in animal models. Some research framework models set up hypoxic cultures over long periods in some cases up to 21 days (Ren et al., 2006) and this reduces the differentiation potential of the cells especially when mesenchymal stem cells are used (Czekanska, 2011). In view of these pitfalls, we aim to propose a model that would not require such long durations to achieve experimental results and plan to use cell lines that are rapidly growing such as MG63 osteosarcoma cells. (Heremans et al., 1978).

This study seeks to create an experimental model to help establish the role for the over-expression of HIF in enhancing osseointegration. The model proposed is aimed at mimicking true hypoxia *in vivo* and cells cultured in the presence of cobalt chloride ($CoCl_2$) grow in a simulated hypoxic micro- environment. $CoCl_2$ is a hypoxia-inducing agent because Co^{2+} replaces Fe^{2+} in haemoglobin, forming deoxygenated haemoglobin and it inhibits HIF-lα aryl hydrocarbon-hydroxylase activity to reduce HIF-lα degradation. Therefore, the features of $CoCl_2$ simulated hypoxia are similar to those of the *in vivo* hypoxic microenvironment (B.

Zhang et al., 2013). It is important to note that the introduction in the use of Cobalt chloride suggested here in this model should not contradict the current views held in the scientific literature on the adverse reaction to metal debris (ARD). This is because of the use of metal on metal or hip resurfacing implants leading to perivascular lymphocytes aggregates with follicles of B and T cells, tissue necrosis, fibrin exudation, endothelial venules and accumulation of macrophages. Due to these adverse biological reactions, the Medical and Healthcare Products Regulatory Agency (MHRA) issued a safety alert in April 2010 and this led to a recall of patients, revision surgery for patients and increased surveillance of patients involving blood metal ions testing annually and cross-sectional imaging (Hart et al., 2011; Matharu, Judge, Pandit, & Murray, 2018). Blood metal ions of cobalt or chromium should not be greater than 7 parts per billion as this is associated with significant soft tissue reactions and failure of metal and metal hips (Benelli, Maritato, Cerulli Mariani, & Sasso, 2019). With this in mind, the use of cobalt chloride here will be as a hypoxic mimetic for the *in vitro* research model, needed for the expression of HIF and concerns about causing arthroprosthetic cobaltism would be negligible.

With the propagation of HIF in hypoxic conditions, the osteogenic bony tissue under these conditions, may have the metabolic and viability potential useful in the physiologic process of osteogenesis. This experimental study aims to answer this question. It is envisaged that the results seen here may add to the body of studies which could serve as a foundation for experimental research modelling in decreasing the incidence of implant loosening and the overall hip surgery revision burden seen not only in sickle cell disease patients with total hip replacements but also in the wider hip arthroplasty patient community.

3.1 MATERIALS AND METHODS

MATERIALS

Cell passaging and Culture Analysis

MG63 human osteosarcoma cells (Sigma Aldrich) were cultured in media composed by volume of 87% Dulbecco's Modified eagle medium (GlutaMax-1) DMEM-(Gibco Life Technologies): 10% Foetal Bovine Serum (FBS)- Sigma: 1% Non-essential Amino-acid (NEAA) 100x-(Sigma Life Science): 1% Penicillin-Streptomycin (P/S)- (Sigma Life Science): 1% Sodium Pyruvate (NaPy) - (Sigma Life Science).

Cell passaging was done with the MG63 cells previously cultured under normoxic conditions- as follows; Cell culture media was aspirated from T75 ml culture flask (Appleton Woods) .

Aspiration was through an aspiration pipette (VWR) which was connected to an aspirator pump (KNF Neuberger). 5mls of Phosphate Buffered Saline(PBS) - (Sigma Life Science) was then added to the well to wash the cells and then carefully aspirated away. 5ml of Trypsin/EDTA solution (Sigma Life Science) was added to the culture flask and then placed in an incubator (Wolf Laboratories) at 37 degrees and 5% CO2 for 5 minutes. This was done inorder to detach the cells from the culture surface in the flask. Once detachment was ascertained under the light microscopy (Nikon Eclipse TS100 Light Microscope) at 100x magnification, then 10ml of culture media which was intially constituted was then added and total voulme transferred to a 50 ml centrifuge tube (Fisher) and centrifuged (DJB Labcare Centrifuge machine) at 1000g for 5 minutes duration.
Once this was done, the supernatant was aspirated leaving the residual cell pellet which was then broken by adding 5 ml of cell media which was then sucked up and down the pipette avoiding the creation of air bubbles.

Cell counting

50µl of the above mixture cell solution is mixed with an equal volume of trypan blue stain (Gibco Life Technologies). Then 10 microliter of this mixture was applied on a haemocytometer fast read slide (Una Health) which was then placed under the light microscope at 10x magnification and then viable cells were manually counted using the a handheld counter tally (Fisher). The fast read haemocytometer slide has grid squares seen under the light microscope and each grid contains 3x3 small squares and viable cells within this a grid is counted as described above. Cells which were non-viable (those through which trypan blue dye had penetrated their cytoplasm) now stained blue were excluded from the count. Three to five sample counts were done and the mean cell number was determined. Cell concentration was then determined using equation 3.1

Cell concentration((cell number/ ml) = Cell count X dilution factor for Trypan blue (2) X 10,000(ml)

Equation 3.1- For calculating the concentration of cell/ml from manual cell counting

From the cell concentration calculated, then the volume of cell solution was then determined in order to yield the required number of cells to be seeded into each well in a six well culture plate based on the well surface area for cell attachment.

Cells were seeded into 6-well plates in cell media with and without Cobalt Chloride ($CoCl_2$) at 100,000 cells per well and cultured under two conditions (normoxic and hypoxic) in the incubator - 20% and 1% oxygen. 3µl of 300mM of Cobalt Chloride solution (Cobalt Chloride $CoCl_2$-(3mM)-University of Bath) was introduced into the experimental samples. The normoxic cells without $CoCl_2$ were used as the control samples. Normoxic samples were incubated in the CO_2 Incubator-(RS Biotech-Model No. 170-200, Copyright 2006) while the incubation for the hypoxic samples was in the Galaxy R CO_2 Incubator- (RS Biotech-Model No. 170-300, Copyright 2006). The incubation period for the cells was 48 and 72 hours. Cell cultures were grown three times (n=3) on three separate consecutive occasions (N=3) (Taylor, Wakem, Dijkman, Alsarraj, & Nguyen, 2010) at 48 hours and 72 hours separately – under the following conditions- 20% normoxia with and without cobalt chloride: 1% hypoxic conditions with and without cobalt chloride. Cobalt Chloride introduced to each well was used to enhance intracellular hypoxia (B. Zhang et al., 2013). All equipment and containers, tubes, pipettes were disinfected with 70% Ethanol solution before being used in the Biohood-Microflow Advanced Biosafety Cabinet (Astec Microflow) to ensure sterility. There was no case of infection of cell culture samples.

Equipment

Applied Biosystems StepOne Plus TM Real Time PCR Sytem Thermal Cycling Block (S/N-272004532)- (Applied Biosystems-Copyright 2010) was used for the qualitative polymerase chain reaction step: The Veriti^{TM} Thermal Cycler (Applied Biosystems 2010) was used for the complementary DNA (cDNA) synthesis step: Synergy HT Multi-detection Microplate Reader-(BioTEK Instruments Inc.-Copyright 2006) with the Microplate Data Collection and analysis software-(BioTEK Instruments Inc.-Copyright 2006- 2008) was used for both the alamar blue assays and the picogreen assays in subsequent studies.
The Galaxy R CO_2 Incubator- (RS Biotech-Model No. 170-300, Copyright 2006) was used to incubate cell cultures under hypoxic conditions while the CO_2 Incubator- (RS Biotech-Model No. 170-200, Copyright 2006) was used for cell cultures under normoxic conditions :Laminar flow biohood (Astec Microflow) - was used for cell passaging under aseptic conditions.

96- well fast thermal cycling plates

MicroAmp™ Optical Adhesive Film (Thermoscientific) and MicroAmp plates (Thermoscientific) were used to place the synthesized complementary deoxyribonucleic acid (cDNA) and the appropriate mix (based on protocol) into the thermocycler for the qPCR procedure.

3.3 METHODS

3.3.1 Ribonucleic acid (RNA) Isolation and extraction

Once cells were incubated under the specified periods- 48 and 72hours, they were washed with 1ml of PBS solution per six well plate. Cell lysis was carried with addition of 600 microliters of RLT lysis buffers per well. The total RNA isolation and extraction was done using the Qiagen RNeasy kit which included an on-column DNase treatment- process which was according to the normal Qiagen RNeasy protocol (QIAGEN, 2012). This was as follows - 1 volume of 70% ethanol was added to equal volume of lysate obtained above in each well. The samples were then transferred to the shredder column and centrifuged for 15 seconds at 10,000 x g and the flow through solution was then placed in the RNeasy Mini spin column. It was then placed in a 2ml collection tube supplied in the kit. This was now centrifuged for 15 seconds at 10,000 x g. The flow through solution was discarded. Then 350µl of RW1 buffer was transferred into the RNeasy column and the lid was closed and centrifuged for 15 seconds at 10,000 x g, the flow through was then discarded. 10µl of DNase 1 stock solution was added to 70µl of Buffer RDD. This was mixed gently by inverting the tube and the 80µl DNase incubation mix was added directly to each RNeasy column membrane and placed on the benchtop (at 20-30ºC) for 15minutes. Once this time had elapsed, 500µl buffer RPE was added to the RNeasy column and centrifuged for 15 seconds at 10,000 x g after which the flow through was discarded. The above step was repeated but this time the RNeasy column was centrifuged for 2 minutes. The RNeasy spin column was then placed in a new 2ml collection tube (supplied in the kit) and then centrifuged at full speed for 1 minute to dry the membrane. Subsequent to this, the RNeasy spin column was now placed into a new 1.5ml collection tube and then 30µl RNase free water was added directly to the spin column membrane. This was kept on the bench top for 1 minute and then the column spin tube was centrifuged for 1 minute at 10,000 x g. The flow through was then put on ice to avoid RNA degradation.

3.3.2 RNA quantification.

RNA quantification was done using the Qubit method - (Qubit 2.0 -Life Technologies) according to manufacturer's protocol (Technologies, 2010).This was done to ascertain the concentration of RNA in each sample and was as follows- two assay tubes for the standards were set up and labelled while one tube for each user sample RNA was set up and labelled as well. The qubit working solution was prepared by diluting the Qubit reagent (provided in the kit) 1:200 in Qubit buffer. 200µl of working solution for each standard and sample. The assay tubes were prepared according to the table below. All assay tubes were vortexed for 2-3 seconds once samples are prepared. The tubes are then inserted into the Qubit™ Fluorometer and RNA quantification readings are taken (Technologies, 2010).

Table 3.1 shows the volume composition for RNA quantification using the Qubit method. (Technologies, 2010)

	Standard assay tubes	Sample assay tubes
Volume of working solution	190µL	198µL
Volume of standard to add	10µL	-
Volume of sample RNA	-	2µL
Total volume in each assay tube.	200µL	200µL

3.3.3 Complementary Deoxyribonucleic acid (cDNA) synthesis

cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Biosystems, 2006). First of all, the reverse transcription (RT) master mix was prepared on ice using the components in the kit as described. A mixture of the following was carried out 2.0μL of 10X RT Buffer: 0.8 μL of 25X dNTP Mix (100mM):2.0 μL of RT Random Primers: 1.0 μL of Multi-Scribe Reverse Transcriptase: 1.0 μL of RNase Inhibitor: 3.2 μL of Nuclease - free water. Once this master mix was prepared then 10 μL of the mix was pipette into each cDNA tube and then 10 μL of RNA sample was transferred into each tube and pipetting up and down twice to mix. The tubes lids were then closed and then centrifuged briefly to spin down the contents and to eliminate air bubbles. The tubes were then placed into the Applied Biosystems Veriti^{TM} Thermal Cycler- Copyright 2008 for the cDNA synthesis.

3.3.4 Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) Quantification

Hypoxic Inducible Factor 1alpha (HIF1 alpha) was used as the experimental gene while 18S and GAPDH were used as the house keeping genes. The coding of the respective primers was as follows-.

Primers-
Hypoxic inducible Factor 1-alpha (Forward and Reverse)-(Sigma Life Science)- with sequence 5'
-AAAGAGGTGGATATGTCTGG (forward); AGTTTGTCTGTCGTTGCTGC (reverse)

18s (Forward and Reverse)-(Sigma Life Science)- with sequence
-AAACGGCTACCACCACATCGAAG (forward): CCTCCAATGGATCCCTCGTTA (reverse)

GAPDH (Forward and Reverse)-(Sigma Life Science)- with sequence
-AAGGTGAAGGTCGGAGTCAAC *(forward): GGGGTCATTGATGGCAACAAT (reverse)*

Expression of HIF1 alpha ,18s and GAPDH RNA was determined by qRT-PCR using the SYBR_ Green PCR mix (Applied Biosystems) and cycle threshold (Ct) values were generated using the Applied Biosystems Step-One plus TM Real Time PCR System Thermal Cycling Block (S/N-272004532)-Applied Biosystems-Copyright 2010. The Ct value is defined

as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct values are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct value the greater the amount of target nucleic acid in the sample). Ct value of < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample. Ct values of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid while Ct values of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination (Beltrame, Cortes, Bandeira, & Figueiredo, 2015; Khan, Choong, Du, & Jovanovi'c, 2013).

In order to quantify gene expression for HIF 1 alpha by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), the 18S and GAPDH values were used to normalize the data. The separate well 2(Delta-Delta Ct) method was used to determine relative fold change expression of HIF 1 alpha. This was normalized to 18S and GAPDH.

3.3.5 Statistical Analysis

Microsoft Excel software version 2016 (Microsoft Inc. Copyright 2016) was used to store and collate the data. Statistics analysis was carried out by using SPSS statistical software (version 24-IBM Cooperation Copyright 1989, 2016). The data, which was non-parametric, was analyzed and differences between the groups ascertained using Kruskal- Wallis H (KWH) test as assumptions for a one -way ANOVA were not met. The data was expressed as mean rank ± SD. A statistically significant result was deemed as p value of <0.05.

3.4 RESULTS

From the results analysed it was clear that there was increased expression of HIF1-alpha using quantitative polymerase chain reaction (qPCR) at 72hours as compared to 48hours (p value<0.05) under the various conditions (*Figure 3.1*). The KWH test was conducted to determine if there were differences in the fold change of HIF between 48 hours and 72 hours. The distribution of the fold change were not similar as assessed by visual inspection of the boxplot. The level of expression of HIF increased from 48hrs (mean rank= 4.60) to 72hrs (mean rank =5.60) but this difference was not statistically significant, $X^2(1) = 0.24$, p =0.625. This means that expression of HIF could be affected by the increased duration of sample culture.

When the level of expression of HIF was assessed against the control reference samples of normoxia without $CoCl_2$, it was noted that there was significant level of expression of HIF at 72 hours duration as compared to the level of expression at 48 hours (*Figure 3.2).* This observation is consistent with the previous assertion of increased expression at 72 hours versus that seen in 48 hours.

It was also explored if there was a difference in the expression of HIF between the normoxic and hypoxic samples. Furthermore, at 48 hours' duration, it was observed that there is decreased expression of HIF between the test samples (hypoxic) when compared with the normoxic samples with or without cobalt chloride *(Figure 3.2)*. This trend was noted to be different when the 72hours samples were observed. Here there was increased expression of the test gene HIF in the hypoxic samples when compared with normoxic samples whether or not they had $CoCl_2$ *(Figure 3.3).* We then checked if there was a statistical significance in the HIF expression between hypoxic and normoxic samples using the KWH test. The values of mean ranks are noted in *Table 3.1.* Distribution of the fold expression data was not similar for both types of samples as confirmed by the visual inspection of the boxplots. The mean rank fold change of HIF in hypoxic samples decreased compared to the normoxic samples but this difference was not statistically significant, $X^2(1) = 0.54$, $p= 0.462$. Therefore, the expression of HIF is only increased with prolonged hypoxia as seen in the 72 hour samples. The expression of HIF in samples with cobalt chloride was seen to be higher than samples without (Figures 3.1 and 3.3). The distribution for the expression of HIF in samples with and without $CoCl_2$ were similar as confirmed by the appearance of the box plots. The expression increased in samples with $CoCl_2$ (mean rank=5.17), compared with samples without $CoCl_2$ (mean rank 4.67), however this was not statistically different, $X^2(1) = 0.067$, $p= 0.796$.

Table 3.2 *Showing the mean rank of fold change expression of HIF under the various sample conditions noted- with and without cobalt chloride, hypoxic or normoxic samples, durations of sample culture (48hrs or 72hrs), and type of reference gene. The significance difference between samples was assessed using the Kruskal Wallis H test as assumptions were not met for ANOVA. The mean rank scores were used to assess for level of significance because the distribution scores for the fold change for the various parameters were disimilar. Significance, p=<0.05*

PRESENCE OF COBALT CHLORIDE ($CoCl_2$)	**MEAN RANK OF FOLD CHANGE EXPRESSION OF HIF**	**SIGNIFICANCE (p value< 0.05)**
With $CoCl_2$	5.17	0.796
Without $CoCl_2$	4.67	
TYPE OF SAMPLE		
Normoxic sample-20% oxygen	5.60	0.462
Hypoxic sample-1% oxygen	4.25	
DURATION OF SAMPLE CULTURE		
48hrs	4.60	0.624
72hrs	5.50	
REFERENCE GENE USED		
18S	4.25	0.773
GADPH	4.75	

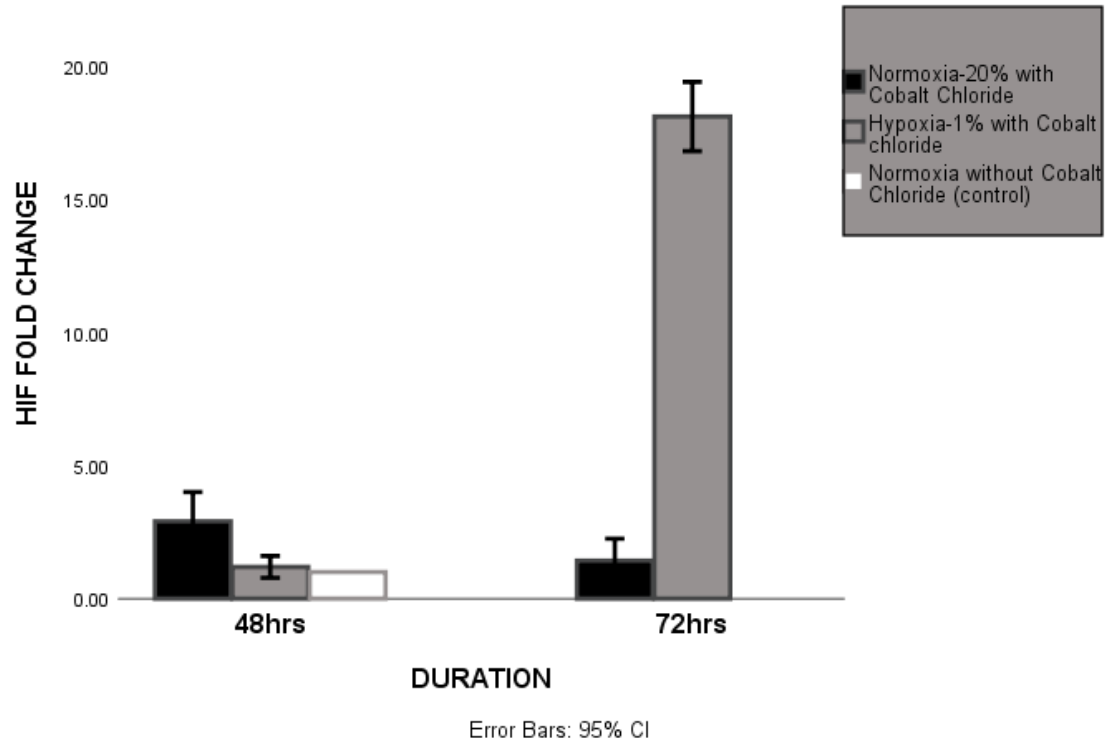

Figure 3.1 *: Shows increased fold change expression of HIF over 72 hours as compared to 48 hours. Mean rank scores confirmed increase but this was not statistically significant p =0.625, p value (>0.05) . The above data was the S.E.M. of three independent measurements on three separate occasions (N=3, n=3).*

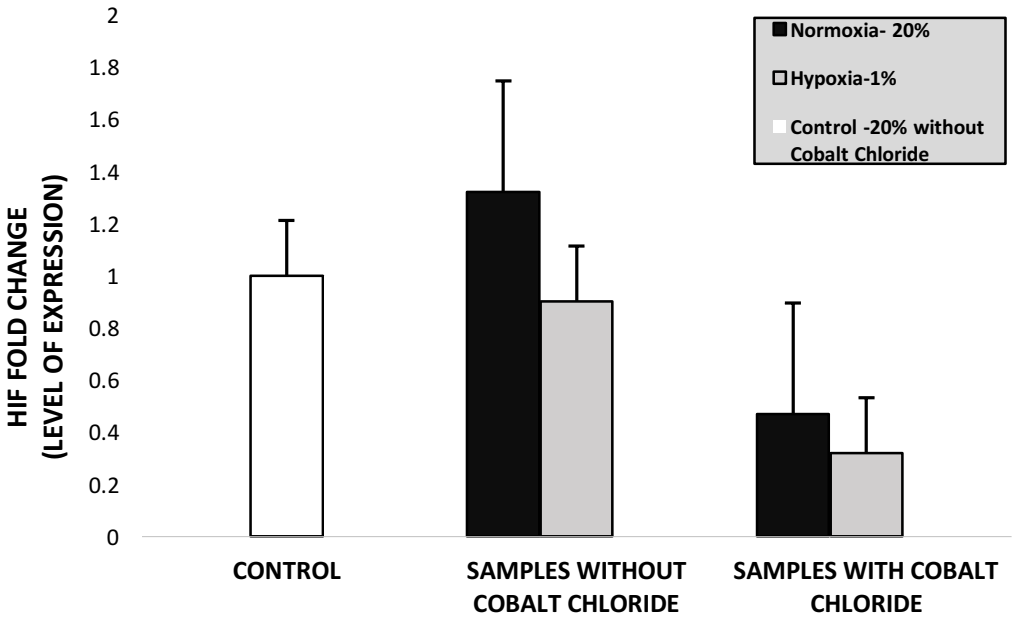

Figure 3.2: *48 hours' fold change- graph of expression of HIF compared with control. This shows decreased expression of HIF when compared with the reference control. There is decreased expression of HIF between the test samples - hypoxia (1%) with Cobalt Chloride ($COCl_2$) when compared with the hypoxic (1%) without Cobalt Chloride ($COCl_2$) samples. There was no statistically significant difference in the mean rank fold change expression of HIF between the normoxic and hypoxic samples with or without $CoCl_2$, p value > 0.05. The above data was the S.E.M. of three independent measurements on three separate occasions (N=3, n=3).*

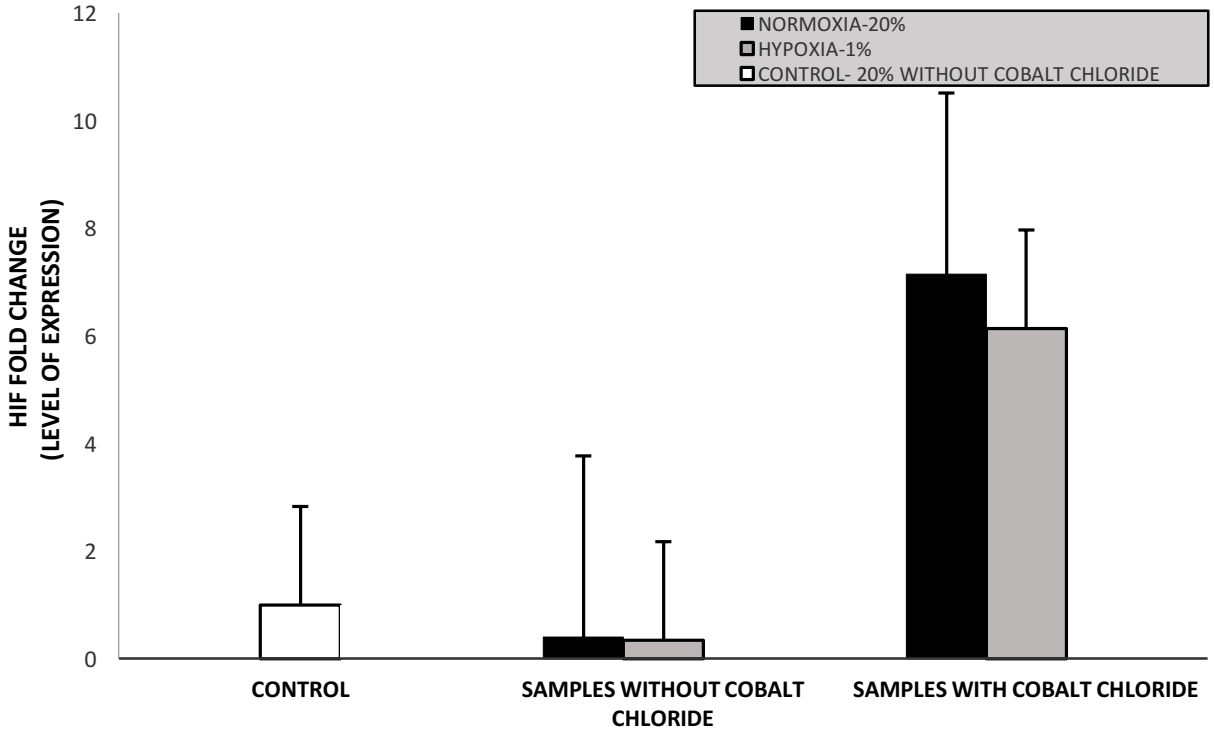

FIGURE 3.3*: 72 hours' fold change- graph of expression of HIF compared with control. This shows increased expression of HIF of test samples hypoxic (1%) with Cobalt Chloride ($COCl_2$) when compared with the reference control. Mean rank score test did not show any statistically significant difference p = 0.462, p value (<0.05). The above data was the S.E.M. of three independent measurements on three separate occasions (N=3, n=3).*

Analysis was done comparing the expression of HIF against the various housekeeping genes -18S and GAPDH. It was noted that there was more expression of HIF with 18S as compared with GAPDH at 48hours with the normoxic and hypoxic samples with $CoCl_2$ but with the hypoxic samples with Cobalt Chloride using GAPDH, there was decreased expression compared with the reference. *(Figure 3.4).* The reverse was the case at 72 hours with increased expression of HIF with the hypoxic samples with $COCl_2$ compared with similar normoxic samples *(Figure 3.5)* at 72 hours' samples expressed HIF more against GAPDH samples when compared to 18s samples. However, there was no statistical difference in the level of expression of HIF between the samples with different reference genes, $X^2(1) = 0.083$, $p = 0.773$.

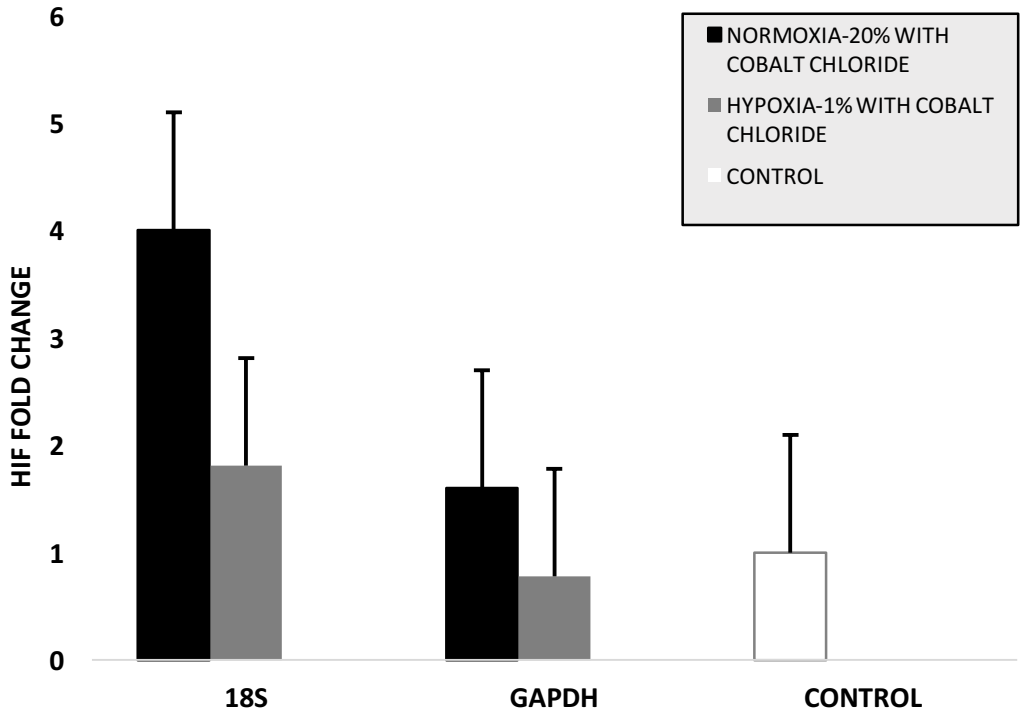

Figure 3.4: *Fold Change of HIF compared to that seen in housekeeping genes (18S and GAPDH) after 48 hours of incubation. There was increased expression of HIF with 18S as housekeeping gene when compared to the control and GAPDH across both norxmoxic and hypoxic samples with $CoCl_2$. The mean rank differences was not statistically significant, p value > 0.05. The above data was the S.E.M. of three independent measurements on three separate occasions (N=3, n=3).*

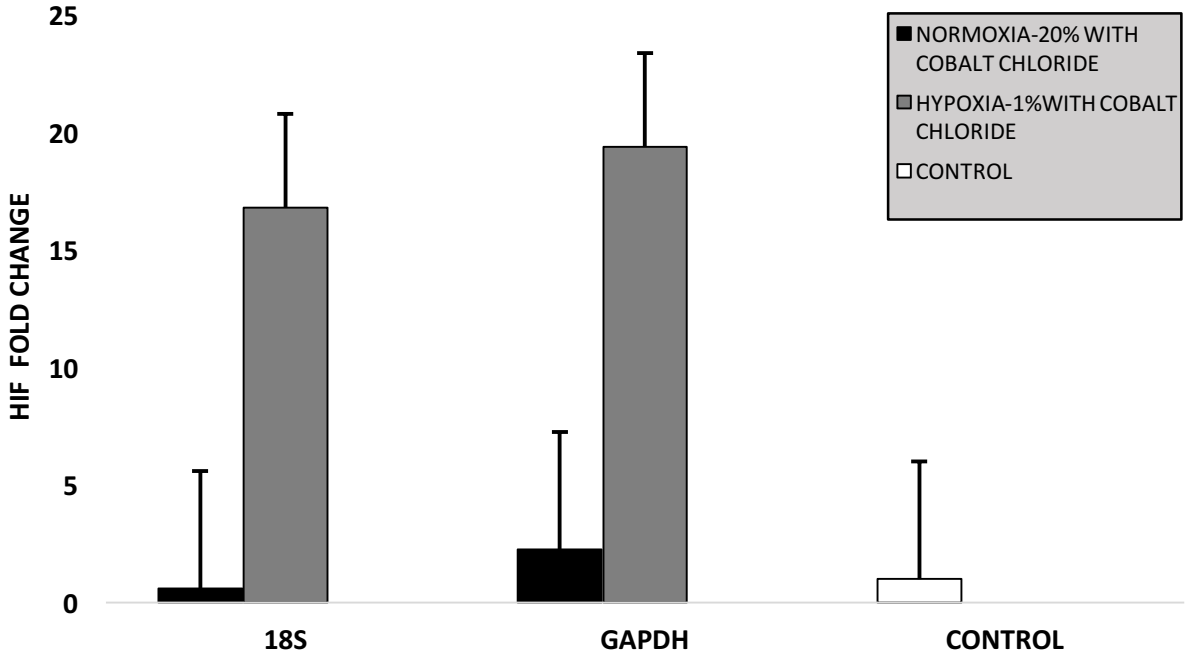

Figure 3.5 *Fold Change of HIF compared to that seen in housekeeping genes (18S and GAPDH) after 72 hours of incubation. There was increased expression of HIF with GAPDH as housekeeping gene when compared to the control and 18S across both norxmoxic and hypoxic samples with* $CoCl_2$. *Mean rank score had a p value >0.05, showing no significant difference. The above data was the S.E.M. of three independent measurements on three separate occasions (N=3, n=3).*

3.5 DISCUSSION

Osteonecrosis of the femoral head (ONFH) is a prevalent complication of sickle cell disease (SCD) that has not been well described in population-based cohort studies. This arises when stiff and abnormally adherent red blood cells repeatedly impair blood flow to susceptible articular surfaces, causing bone infarction at the epiphyseal plates and early onset degenerative arthritis (Adesina, Brunson, Keegan, & Wun, 2017). The resultant hypoxia is the physiologic sequelae by which the microcellular bony environment adjusts. Adaptation to low oxygen tension (hypoxia) in cells and tissues leads to the transcriptional induction of a series of genes that participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival. The primary factor mediating this response is the hypoxia-inducible factor-1 (HIF-1), an oxygen-sensitive transcriptional activator (Ke & Costa, 2006). Several studies including this one has shown that HIF-1 can be upregulated dependently in response to hypoxia in osteoblast cells (Sun & Peng, 2015; Wan et al., 2010). The results in the present study are comparable to established experiments in the literature. This study confirms that prolonged hypoxia caused over expression of the hypoxic inducible factor. Although many experimental studies have looked at pathologic conditions including cancerous ones to establish the role of HIF in their pathogenesis (Carmeliet & Jain, 2000; Pralhad, Madhusudan, & Rajendrakumar, 2003), conditions such as sickle cell bone disease have little or no studies attributed to it, highlighting the role of HIF in the disease sequelae. Kyoung et al. speculated in their study that tissue hypoxia, which occurs in vasocculsion in SCD, infarction, and stroke, up-regulates the expression of chemokines, i.e., IL-8 in vascular endothelium (K. S. Kim, Rajagopal, Gonsalves, Johnson, & Kalra, 2006). However, the role of HIF was not highlighted in their study. This study however aimed to establish a basis for HIF over expression and contribute to the body of knowledge out there with regards angiogenesis and osteogenesis, which are consequences from the upregulation of the HIF pathway.

Over the past decade, work from many laboratories have indicated that hypoxic microenvironments contribute to cancer progression by activating adaptive transcriptional programs that promote cell survival, motility, and tumor angiogenesis. Tumor cells residing closer to blood vessels are relatively well oxygenated, whereas those cells at more distant sites are hypoxic. Stabilization of HIF-α proteins in these cells stimulates the expression of numerous target genes encoding factors that mediate adaptation to hypoxic stress (Keith & Simon, 2007). These studies have proposed ways by which inhibiting the HIF pathway could help form therapeutic basis for cancer treatment. The results from this study showed that overexpression of HIF cascade of reactions could be a platform for osseointegration, which in this case is not an inhibition but a propagation of HIF. The role of the hypoxic inducible

factor in neoplasia pathogenesis has helped us draw some lessons in understanding its role in enhancing bone implant integration through its augmented expression as established in this study.

Cobalt Chloride was introduced into MG63 cell culture samples as a mimetic for hypoxia. This was necessary to ensure adequate further mimicking of *the in vivo* microcellular environment seen in the pathologic process of avascular necrosis akin to that in sickle cell disease bone. In the laboratory such as in this case, with high-density cell culture methods, problems of oxygen deprivation become unavoidable with culture volumes exceeding only a few liters. Sparging of air or oxygen into mammalian cell culture growth media is not always suitable. It causes either damage to the cells by rupturing the membranes, or foaming and sometimes both (Fleischaker & Sinskey, 1981). In addition, studies have shown a discrepancy in the hypoxic modelling and partial pressure of oxygen is over stated. Established literature confirms that the partial pressure of oxygen (PaO_2) in the bone marrow is 6.3% while in cells it is 1% (Carreau, El Hafny-Rahbi, Matejuk, Grillon, & Kieda, 2011). Although there is no documented evidence of measurement of PaO_2 in SCD patients as far as we are aware, there have been studies suggesting mathematical and computational models which could advance this (Filas, Shui, & Beebe, 2013). However, it is assumed that with low haemoglobin levels in these patients, the PaO2 in these patients could be much lower than in normal patients hence the need to ensure and aim for pathologic hypoxia in this model as seen in SCD patients. For this to be achieved with this experimental model - in other words simulating the *in vivo* conditions seen in SCD bone marrow, Cobalt Chloride was introduced to cell culture samples in order to ensure this. $CoCl_2$ inhibits HIF-lα aryl hydrocarbon-hydroxylase activity to reduce HIF-lα degradation and so the features of Cobalt Chloride-simulated hypoxia are similar to those of the *in vivo* hypoxic microenvironment (B. Zhang et al., 2013). This could be the strength and strong selling point for this model.

Furthermore, in designing the model for this experimental study, qPCR was used for the amplification of the test gene which was achieved after the cycle threshold (CT) values were normalized to two housekeeping genes-18S and GAPDH. Reverse transcriptase qPCR has been noted to be fast and delivers reliable and cost-effective results when it comes to gene expression. However, Dijkstra et al in their systemic review of published qPCR experiments noted that most experiments only used one reference gene and this reduces the validity of such experiments (Dijkstra et al., 2014a). We have used two reference genes in this study to ensure adequate validity but excellent validity could be desired if more than two reference genes were used. The results deduced here shows inconsistent results with decreased expression of HIF at 48 hours and increased expression at 72 hours when GAPDH alone is used as a reference gene. Although this trend is not seen with 18S as the reference gene,

these results further support the principle of normalization of the HIF expression with more than one reference gene to ensure validity.

It is important to note that there were limitations with this model. Kitchen et al in their own study describe a method of estimating the components of biological variation and technical noise directly from qPCR measurements. This was achieved through the exploitation of a small number of biological and technical replicates at each stage of the sample processing procedure. These stages being the inter-subject, inter-sample, inter-RT, and inter-qPCR. These biological replicates form a pilot study to the larger main prospective investigation. (Kitchen, Kubista, & Tichopad, 2010). In the present study, this extensive and rather expensive method was not employed and hence this could be a limitation to the study. Another limitation to this study was that the results derived from the model developed was not validated using another test method. Real-time qPCR was used to determine the expression of HIF in the test samples and to further validate the results, enzyme linked immunosorbent assay (ELISA) could be used to assess the expression of HIF. The high sensitivity of qPCR allows for the detection of very low amounts of protein expression (Gomez et al., 2002). This formed the basis for its use in this model and in other experimental work but the novelty and usefulness of the model with the use of cobalt chloride could help lend more credence to this model by validation with ELISA (Dijkstra, van Kempen, Nagtegaal, & Bustin, 2014b). ELISA is a protein-based method while qPCR is DNA-based, and so there has been well-documented facts on the pros and cons of their use. ELISA is a quicker and easier method to carry out with less tissue handling and with no problems of RNA extraction and cDNA synthesis errors to overcome. However, qPCR is less expensive and more specific (Adam T. Perestam, 2017). Some researchers have used both methods to help assess samples in order to aid validation of their results (Bensalah, 2019; Nance, Riederer, Zubkowski, Trudel, & Rhodes, 2010). This was not the case in this study and so an important limitation to take note of.

3.6 CONCLUSION

This study sought to create an *in vitro* model to help establish the over expression of HIF in enhancing osseointegration. An *in vitro* model was developed in which MG63 osteoblast cells were cultured under hypoxic and normoxic conditions at 48 and 72 hours' durations. The over expression of HIF was achieved within a few days (72 hours) with the introduction of Cobalt Chloride at the concentration of 300mM, which is a mimetic for hypoxia and making the investigation of avascular necrosis in pathologic conditions such as sickle cell

possible. It was noted that there was more over expression of HIF at a longer duration of culture at 72 hours.

This model was noted to be novel in its use in this work as it mimics the pathologic environment in which femoral implantation in THR takes place *in vivo.* To the best of the author's knowledge, no other study has highlighted this. However further questions arise pertaining to the metabolic viability of MG63 cells in which HIF is over expressed; could these osteoblastic cells in severe hypoxia have the functional metabolic activity to initiate satisfactory osseointegration? It is therefore crucial that further studies be done in order to scientifically establish this microcellular phenomenon and hence the need for the next study outlined in the next chapter of this work.

CHAPTER FOUR

INVESTIGATING CELLULAR VIABILITY OF MG63 CELLS WITH COBALT CHLORIDE IN AN HYPOXIC ENVIRONMENT- A PROPOSED *IN VITRO* MODEL

4.0 INTRODUCTION

Bone formation requires the contribution of osteogenic (bone-forming) connective tissue progenitors (CTP-Os). These progenitors represent a heterogenous population of cells in the bone marrow that are capable of proliferating and differentiating into one or more connective tissues (Heylman et al., 2014). In sickle cell, the pathogenesis of the disease condition leads to bone marrow hyperplasia and a sclerotic medullary canal (Azam & Sadat-Ali, 2016). This is the suboptimal bone environment that femoral implants are iinserted for those needing THR and this again does not encourage osseointegration. However, having suggested in the previous chapter that the proposed model could express the hypoxic inducible factor which invariable enhances osteogenesis, it is therefore important to ascertain if this osteogenic process in the presence of HIF expression could yield osteocytes with significant enough metabolic bioviability. This is one objective we seek to explore with an *in vitro* model that could help investigate this.

The prevalence of osteonecrosis in patients with sickle cell disease is very high, approaching 50%. As demonstrated by various studies, progression to collapse is almost universal and the results of total hip replacement are much more problematic than in patients with other types of arthritis (Luck, 2006). Implant loosening rate is a major concern in these patients. It is hypothesized that the sequel of the disease, which includes vasocclusive crisis leading to repetitive bone infarction from hypoxia, could be the reason for lack of osseointegration of the prosthesis (Bishop et al., 1988; Moran, Huo, Garvin, Pellicci, & Salvati, 1993). There is still a paucity of studies aimed at highlighting this in these types of patients.

Angiogenesis and osteogenesis are tightly coupled during bone development and regeneration. Mesenchymal cells in the developing stroma elicit angiogenic signals to recruit new blood vessels into bone. Reciprocal signals, likely emanating from the incoming

vascular endothelium, stimulate mesenchymal cell specification through additional interactions with cells within the vascular stem cell niche. The hypoxia-inducible factor-1 alpha (HIF-1) pathway has been identified as a key component in this process (Wan et al., 2010). It was obvious from the previous chapter that the HIF1 alpha protein over expressed in the hypoxic microcellular environment could be the experimental platform for further research into enhancing osseointegration in addressing the problem of prosthetic implant loosening in sickle cell disease patients. The model proposed still raised questions. One of such questions has to do with the lack of clarity on the role of cobalt chloride. Some studies have shown that the stimulatory effect of hypoxia on vasoactive endothelia factor VEGF can be reproduced by cobalt chloride. In addition, cobalt has been found to be a potent stimulator of VEGF *in vivo* (Minchenko, Bauer, Salceda, & Caro, 1994).

The model in the previous chapter like other documented studies, highlighted the role of $CoCl_2$ as a hypoxic mimetic ensuring true cellular hypoxia in order to mitigate well documented physiologic false positive hypoxic measurements in cell culture. With this in mind there is however no documented evidence in the literature of how much cellular metabolic activity takes place in hypoxic conditions when human MG63 cells are subjected to enhanced hypoxia in the presence of cobalt chloride. This study is aimed at assessing the level of osteoblastic cellular proliferative bioactivity in order to give credence to the earlier proposed experimental model investigating improved osseointegration in avascular necrosis based on over expressed HIF transciption protein in an hypoxic environment. It is further speculated that through this study, an experimental model design in co-operating cobalt chloride in the microcelluar *in vivo* osteoblastic environment could be beneficial in developing a useful research model and may be effectual in adding to the little body of research evidence directed at enhancing osseointergation in SCD patients.

4.1 METHODOLOGY

Cell culture and passaging

MG63 human osteosarcoma cells (Sigma Aldrich) were cultured in cell media composed by volume of 87% Dulbecco's Modified eagle medium (GlutaMax-1)DMEM- (Gibco Life Technologies): 10% Foetal Bovine Serum (FBS) - Sigma: 1% Non-essential Amino-acid (NEAA) 100x-(Sigma Life Science): 1% Penicillin-Stretomycin (P/S)- (Sigma Life Science): 1% Sodium Pyruvate (NaPy)- (Sigma Life Science). Cells were seeded into 6-well plates at 100,000 per well under two conditions in the incubator- 20% and 1% oxygen with 3µl of 300mM of Cobalt Chloride. The normoxic cells without cobalt chloride were used as the control samples. All cell passaging was carried out under strict sterile conditions in the Biohood. The incubation period for the cells was 48 hours and 72 hours. Cell cultures were grown three times (n=3) on three separate consecutive occasions (N=3) at 48 hours and 72 hours separately under the following conditions; - 20% normoxia with and without cobalt chloride: 1% hypoxic conditions with and without cobalt chloride. Cobalt chloride introduced to each well was used to enhance intracellular hypoxia due to reasons stated previously.

Alamar Blue Assay to Assess Cellular Viability

Following the duration of cell culture, Alamar Blue Assay was employed to assess MG63 cellular viability. A stock solution of 0.15 mg/mL Resazurin/Alamar blue in Phosphate Buffered Saline (PBS) at pH 7.4 was prepared according to documented protocol. Stock solution was filtered and protected from light and stored at 4 degrees centigrade.

After cell culture, 60µl of Alamar blue stock solution was added to each well without decanting the cell media and allowed to incubate for two hours protected from light. Fluorescence was then read at 560/590 nm using the Synergy HT Multi-Detection Microplate Reader- (BioTEK Instruments Inc.-Copyright 2006) - with the Microplate Data Collection and Analysis Software- (BioTEK Instruments Inc.-Copyright 2006- 2008). The Alamar blue assays were carried out in triplicates (n=3) on three separate days (N=3).

Picogreen Assay

Picogreen assay (Gibco Life Technologies) was used in this study to quantify the amount of DNA from the cultured cells, as dependent on cell number. Once the Alamar blue assay fluorescence signals was read, the cell culture media and Alamar blue reagent was aspirated carefully. Then each well of the cell plate was washed with PBS and then aspirated. 1X TE (Tris EDTA) buffer was added to each well and each well plate was then allowed to freeze/thaw under temperatures – 80 degrees and 25 degrees. This was done three times to release the DNA from the cells.

The Picogreen reagent was then diluted in 1X TE buffer solution in a 1:200 ratio. Then 75µl of mixture with equal amount of prepared DNA/TE buffer put in a 96 well plate and absorbance readings taken. In addition, samples of known DNA concentrations were prepared and mixed with Picogreen reagent and fluorescence readings taken in order to generate DNA versus fluorescence standard curve (*Figure A20).* Furthermore, in order to generate a standard curve of cell number versus Picogreen absorbance samples from known cell number also underwent the thaw/freeze sequence and mixed with the Picogreen reagent after which fluorescence readings were taken and the curve generated *(Figure A21).* Both standard curves were generated from triplicate assays (n=3) on three separate days (N=3) corresponding to the Alamar blue assays. The absorbance readings were then taken from the fluorospectrometer at 480nm/520 nm excitation.

The standard curve of fluorescence/number of cells was used to determine how many cells were in the experimental samples. The data was normalised, as there was different number of cells in the experimental samples. This was done by dividing the average Alamar blue signal for each condition by the average number of cells for each condition and so signal (absorbance) per cell was determined.

Statistical Analysis

The fluorescence ratios were calculated to evaluate the signals and data collated using Microsoft Excel software version 2016 (Microsoft Inc. Copyright 2016). Statistics analysis was carried out by using SPSS statistical software (version 24-IBM Cooperation Copyright 1989, 2016). The data are expressed as mean ± SD and analysis was performed to indicate a statistically significant result p value of <0.05. The one-way ANOVA test (post Hoc Dunnett T3) was used and non-parametric data was analyzed using the Kruskal Wallis test to check for level of significance, p value <0.05 was deemed significant. Multiple Regression was used to assess the level of prediction for absorbance per cell. The assumptions for multiple

regression analysis as confirmed by the visual inspection of the histogram and normal P-P Plot *(Figure A22)* and the assumption of homoscedasticity of the data was fulfilled. A three-way ANOVA was also carried out to check for the overall effect of duration of culture, type of sample (hypoxic or normoxic) and presence of cobalt chloride on the absorbance per cell (Statistics, 2017b).

4.2 RESULTS

The results from the study were aimed at assessing osteoblastic cellular viability and proliferation under the various conditions highlighted previously. It was observed from the Alamar blue assay that there was statisitically difference in the absorbance per cell in samples cultured under the hypoxic conditions with $CoCl_2$ chloride when compared with the control after 48hours *(Table 4.1,Figure 4.1).* The mean signal per cell was more in the experimental sample (hypoxia with cobalt chloride- 0.01, p value <0.05) compared with the control (normoxia without cobalt chloride - 0.0007) meaning increased cellular viability capacity despite the condition of hypoxia and presence of $CoCl_2$. It was also noted that there was significant difference in the absorbance per cell with samples cultured under normoxia with $CoCl_2$ (0.005, p value <0.05) when compared with the control *(Table 4.1).* The experimental samples also showed decreased viability and proliferation. This is expected due to the presence of cobalt chloride in normoxic conditions.

The results was different after 72 hours duration. It was noted that there was increased absorbance per cell under hypoxia with cobalt chloride (0.0025, p value >0.05) but there was no statisitically significant difference in the flouresence absorbance per cell when compared with the control of normoxia without chloride and the same is the case with increased absorbance per cell under hypoxia without chloride compared with the control, 0.002 (p value >0.05) and this difference was not significantly different *(Table 4.2,Figure 4.2).* Although there was observed increased absorbance per cell in the hypoxic samples with $CoCl_2$ as compared to the normoxic cell samples with cobalt chloride (0.001, p value >0.05), this was not statisitically significant *(Figure 4.2).* Furthermore as seen in the 48 hour study, there was decreased absorbance per cell in samples cultured under normoxia without $CoCl_2$ as compared with similar samples with $CoCl_2$. The reverse was the case in the normoxic 72 hour samples. This could be due to the apoptosis effect of Cobalt Chloride enhanced in the 72 hour samples due to the increased duration. The afore mentioned trend is however not seen in the 72 hour hypoxic set of samples because of the expected lack of enhanced

expression of the hypoxic inducible factor in hypoxic samples without cobalt chloride. Here, there is decreased osteogenesis and the decreased absorbance in cells.

The three way ANOVA was used to determine the overall effect of the duration of culture, type of sample (either normoxic or hypoxic), presence of cobalt chloride or not on the absorbance per cell. There was no statistical interaction between these three parameters on the absorbance per cell, $F(1,64) = 2.311$, $p = 0.133$ (p value signifcant at <0.05) (Field, 2013; Statistics, 2017b).

A multiple regression was run to predict abosrbance per cell from the type of sample (normoxic or hypoxic); duration of culture (48hrs or 72hrs) and the presence of cobalt chloride (present or not). There was independence of residuals as assessed by a Durbin - Watson statisic of 1.5. There was homoscedasticity as assessed by visual inspection of grouped scattered plots *(Figures A23,A24,A25).*

The multiple regression model statistically significantly predicted absorbance, $F(3,68) = 18.902$, $p<0.001$, $R^2 = 0.455$. Two of the variables (type of sample and duration of culture added significantly to the prediction, $p<0.001$: presence of cobalt chloride did not predict signficantly the absorbance per cell, p value =0.5 (Statistics, 2015b). The regression coefficients and standard errors can be seen in *Table 4.3*

Table 4.1- *Showing multiple comparison of absorbance per cell at 48 hours under the different experimental conditions.*

TYPE OF SAMPLE	MEAN DIFFERENCE	SIGNIFICANCE LEVEL (p value<0.05)
Normoxia with Cobalt Chloride Compared with Normoxia without Cobalt Chloride	0.0005*	<0.05
Normoxia without Cobalt Chloride Compared with Hypoxia with Cobalt Chloride	0.0007*	<0.05
Hypoxia without Cobalt Chloride Compared with Normoxia without Cobalt Chloride	0.024	0.595
Hypoxia with Cobalt Chloride Compared with Hypoxia without Cobalt Chloride	0.023	0.598

** Significant level at 0.05 level (2-tailed)*

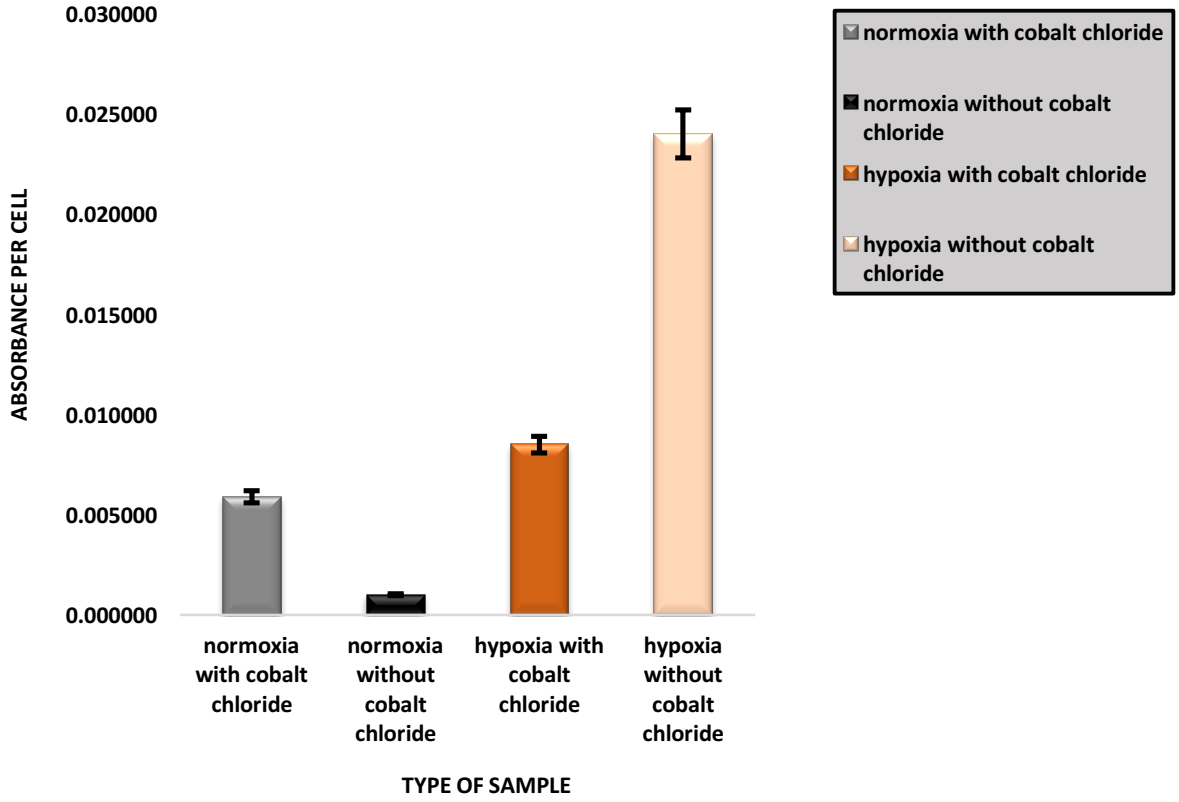

FIGURE 4.1- *Showing absorbance per cell under various experimental conditions at 48 hours with normoxia without $CoCl_2$ being the control. There was significant difference in the absorbance per cell under the hypoxic conditions with Cobalt Chloride compared to the control- normoxia with $CoCl_2$-at 48hours: Kruskal Wallis test used to determine p value < 0.05. One way ANOVA (post hoc. Dunnetts test) showed significant difference in absorbance per cell between control sample and normoxia with $CoCl_2$,p = value <0.05. The above data was the S.E.M. of three independent measurements on three separate occasions (N=3, n=3).*

Table 4.2- *Showing multiple comparison of absorbance per cell at 72 hours under different experimental conditions.*

TYPE OF SAMPLE	MEAN DIFFERENCE	SIGNIFICANCE LEVEL (p value<0.05)
Normoxia with Cobalt Chloride Compared with Normoxia without Cobalt Chloride	-0.0003	0.959
Normoxia without Cobalt Chloride Compared with Hypoxia with Cobalt Chloride	-0.0009	0.712
Hypoxia without Cobalt Chloride Compared with Normoxia with Cobalt Chloride	-0.0007*	0.05
Hypoxia with Cobalt Chloride Compared with Hypoxia without Cobalt Chloride	$4.4X10^{-5}$	0.971

* *Significant level at 0.05 level (2-tailed)*

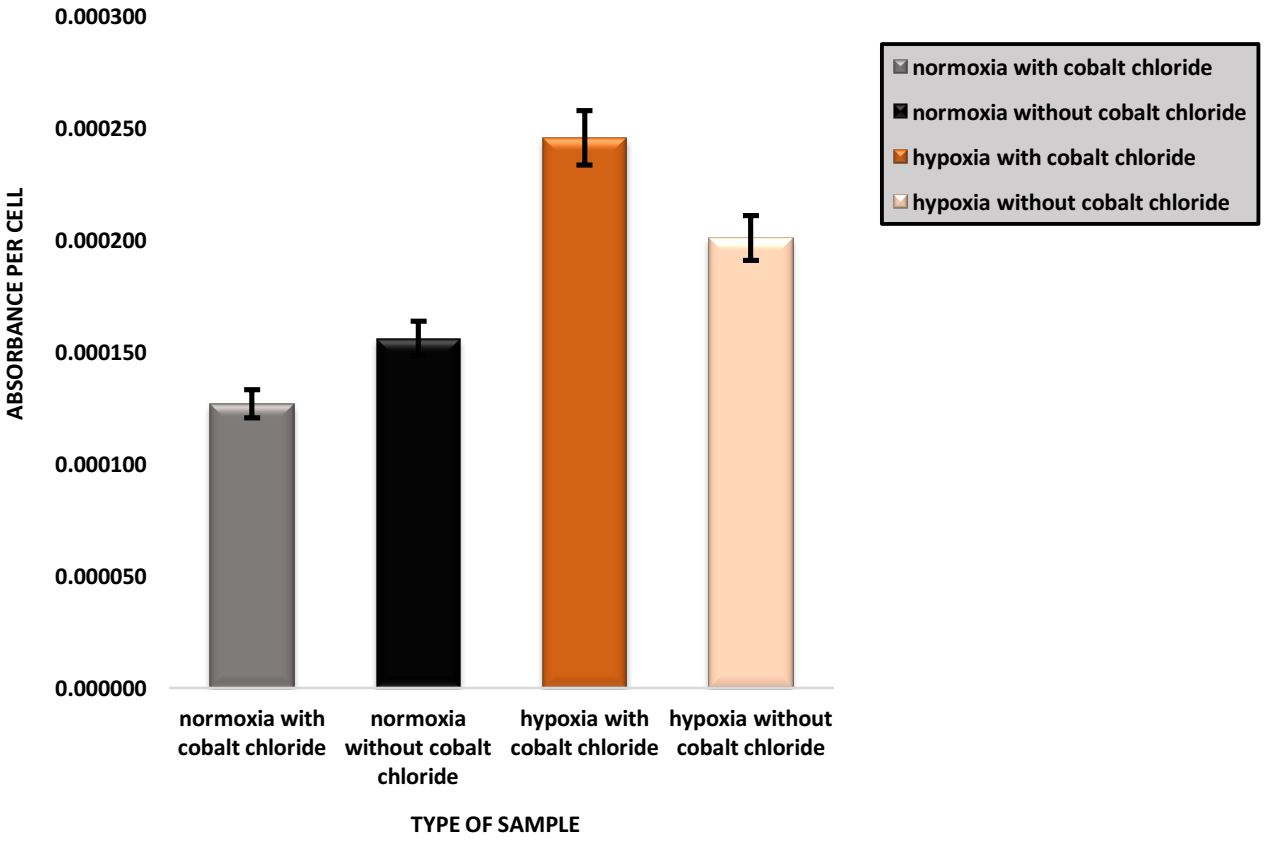

FIGURE 4.2- *Showing absorbance per cell under various experimental conditions at 72 hours with normoxia without $CoCl_2$ being the control. There was no significant difference in the absorbance per cell under the hypoxic conditions with Cobalt chloride compared to the control- normoxia without CoCl-at 72hours: Kruskal Wallis H (KWH) test used to determine p value > 0.05. One way ANOVA (post hoc. Dunnetts test) showed significant difference in absorbance per cell between the hypoxic without $CoCl_2$ samples and normoxia with $CoCl_2$ samples, p value <0.05. The above data was the S.E.M. of three independent measurements on three separate occasions (N=3, n=3) .*

Table 4.3- *Table showing summary of multiple regression analysis highlighting the predictors of absorbance per cell. Duration and type of sample were noted to be significant predictors.*

VARIABLE	B	$S.E_B$	β
Intercept	-0.001	$1.23x10^{-5}$	
Duration	$1.19x10^{-5}$	$1.8x10^{-5}$	0.579*
Type of sample (hypoxic/normoxic)	$6.9x10^{-5}$	$1.8x10^{-5}$	0.340*
Presence of cobalt chloride	$-1.23x10^{-5}$	$1.8x10^{-5}$	-0.675

*NOTE- * level of significance p <0.001; B - Unstandardized regression coefficient ; SE_B – Standardized error of coefficient; β – Standardized coefficient*

4.3 DISCUSSION

It has been established in the literature that the endothelial cell response to hypoxic stress can result in two different consequences in the surrounding tissues, depending on the duration of the exposure: short-term exposure causes physiological and reversible modulation of vascular tone and blood flow; chronic hypoxic stress results in irreversible remodelling of the vasculature and surrounding tissues, with smooth muscle proliferation and fibrosis. This dichotomy of responses to hypoxia may explain in part, both the acute and chronic pathophysiological sequelae of diseases characterized by regional hypoxia, including atherosclerosis, pulmonary hypertension, sickle cell disease and systemic sclerosis (Faller, 1999). However, it has been noted that these responses could also vary depending on different sensations and transduction of hypoxic and hyperoxic signals (Abidia, 2000). The results from our study sought to further establish and confirm the former assertion through the model employed. MG63 cells cultured under various hypoxic conditions and with a mimetic agent such as Cobalt Chloride seeks to mimic the *in vivo* pathologic microcellular environment seen in sickle cell disease avascular necrosis. It was observed in the experimental samples that there was increased cell viability at 48 hours for the hypoxic with $CoCl_2$ samples. This is consistent with results seen in other studies, which highlights the role of HIF where hypoxia was noted to increase adhesion of MG63 spheroids and enhances their ability to spread into the surrounding fibroblast cell culture (Indovina, Rainaldi, & Santini, 2008). Grayson et al. hypothesized from the results in their study that the human mesenchymal cells cultured in hypoxic conditions displayed significantly improved expansion characteristics while maintaining their multi-lineage potential (Grayson, Zhao, Bunnell, & Ma, 2007). However, this study further highlights the role of low partial pressure of oxygen (1%) by exaggerating the cellular level of hypoxia with the introduction of Cobalt Chloride, which to the best of our knowledge no other study has done when doing similar investigations.

In this study the fact that short durations of hypoxia was employed (48 hours and 72 hours), results are similar to that seen in other studies where the experimental model involved culturing cells for up to 28 days (Potier et al., 2007). This may be akin to enhanced pathologic hypoxia but the introduction of Cobalt Chloride covers for this limitation and further authenticates the model in this study. However, there may be some variation in the results due to the increased apoptotic effect of $CoCl_2$ and this was seen with the different absorbance per cells in samples cultured for 72 hours. There was no statistical difference in the level of cellular viability between the experimental samples and the control. This was not the case with the 48 hours' samples showing significant difference. Although there was increased viability compared to the control at 72 hours this was not statistically significant.

There are credible reasons for this. There is established literature evidence describing the genotoxicity and cytotoxicity of cobalt ions behaviour in the cell culture medium of the nanomaterial studied (Ponti et al., 2009). The longer exposure to Cobalt Chloride could be responsible for the difference in results. This observation however confirms other hypotheses noted in various studies that $CoCl_2$ prevents tumor formation in osteosarcoma cells and this could form the therapeutic basis for treatment of this neoplasia (B. Zhang et al., 2013). However, in contrast the regression analysis does show that $CoCl_2$ is not a significant predictor of absorbance per cell unlike the duration of cell culture and whether or not the sample was hypoxic or normoxic. This could explain the variation in cellular viability noted in the 48 and 72 hour samples noted earlier.

The experimental model employed in this study involved the Alamar blue assay. It was used to evaluate the MG63 cells *in vitro* for cellular viability and metabolic potential under the various experimental conditions, which is similar to the *in vivo* pathologic sickle cell environment. Alamar blue is a cell viability assay reagent that contains the cell permeable, non-toxic and weakly fluorescent blue indicator dye called Resazurin. Alamar Blue is a useful non-toxic alternative to the commonly used MTT cell viability assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (O'Brien, Wilson, Orton, & Pognan, 2000). Alamar Blue (AB) is a water-soluble dye that has been previously used for quantifying *in vitro* viability of various cells and because it is extremely stable and more importantly non-toxic to the cells, continuous monitoring of cultures over time is possible. Mainly for this reason, this test has been considered superior to classical tests for cell viability (Ahmed, Gogal, & Walsh, 1994; Fields & Lancaster, 1993). The Alamar Blue assay provides accurate time-course measurements. It has high sensitivity and linearity, involves no cell lysis, is ideal for use with post-measurement functional assays, is flexible and it can be used with different cell models. In addition, it is scalable and can be used with fluorescence and/or absorbance-based instrumentation platforms, and finally, it is non-toxic, non-radioactive and is safe for the user and the environment (Rampersad, 2012). Al-Nasiry et al established in their study that the use of Alamar blue assay was a reliable method of assessing the viability, migration and invasion of choriocarcinoma cells and so can be used as a model for invasive trophoblasts. Such models may prove valuable in the continuing search for assays which mimic the *in vivo* environment (Al-Nasiry et al., 2007). This present study also sought to achieve a similar goal which is to mimic the *in vivo* pathologic environment seen in sickle cell avascular pathology. The results from this study using the Alamar blue as a detector of cellular viability and proliferation highlights that osteoblasts cultured under hypoxic conditions in the presence of cobalt chloride could be a research platform, which establishes that the role of HIF could even be the foundation for improving

osseointegration in future implant design. This study highlights the experimental model used here as a possible research tool for further prosthetic implant design needed to address the matter of increased failure rate seen in sickle disease patients with total hip arthroplasty. There are some limitations to this model though, of which we are fully aware. The lack of validation of the results from this study using a concurrent study with the use of other assay methods to check cellular viability such as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) cell assays would give more credence to this model. Though the MTT assay is less sensitive (Hamid, Rotshteyn, Rabadi, Parikh, & Bullock, 2004), this assay compares with the Alamar blue assay and so comparison results would be helpful. *In vivo* animal studies may be useful to help validate this model in the future. Furthermore, there was no variation in the concentration of the $CoCl_2$ used in order to check the cell response over intermediate lower concentrations of the mimetic agent. Bearing in mind the cytotoxicity of $CoCl_2$, varying reduced concentrations compared to that used, could have improved cellular viability.

4.4 CONCLUSION

This study aimed to assess the level of osteoblastic cellular proliferative bioactivity in order to give credence to the earlier proposed experimental model developed to investigate improved osseointegration in avascular necrosis, based on over expressed HIF transcription protein in a hypoxic environment. It was concluded that in the presence of low oxygen concentration (1%), osteoblasts as represented by MG63 cells are physiologically viable and so have functional potential. It was noted under prolonged duration of hypoxia (72 hours) and low oxygen saturation levels (1%) mimicking *in vivo* sickle cell bone marrow pathologic conditions; these cells did have some level of metabolic function. Despite the effects of $CoCl_2$, which was used here to exaggerate the level of hypoxia, MG63 cells maintained some bioactivity and function. In this study, it was established that despite hypoxia, osteoblasts still maintain functional cellular function and this knowledge could act as a novel step in further investigating and improving bone-implant bonding in SCD patients.

In addition, we hypothesized that the Alamar blue assay used to investigate this phenomenon although not novel in its use could be adapted along with other assays in further research to investigate bone implant osseointegration. Adequate validation work on this model could help make it more effective in wider research involving the development of more suitable *in vitro* models that could mimic *in vivo* pathologic conditions such sickle cell bone disease.

CHAPTER FIVE

GENERAL DISCUSSION AND IMPLICATION OF THIS RESEARCH STUDY

5.0 SUMMARY OF THESIS

The main objective of this research work was to propose an experimental model which could be useful in further investigating osseointegration in the presence of a pathologic bony environment such as avascular necrosis as seen in SCD patients. A review of the literature was done highlighting the nature of SCD, the mode of failure of femoral implants in THR and its problems in these patients. Furthermore the role of the hypoxic inducible factor (HIF) was also brought to the fore and its effect as a useful protein in enhancing osseointegration was further explored. The questions raised in the review formed the basis for studies carried out in the research. It was first of all concluded through the clinical study work (chapter 2) that the shape of the proximal femur in sickle cell disease patients may have an effect on femoral component implant loosening. Also bearing in mind the sclerotic and hypoxic pathologic state of femoral bone marrow where the prosthesis is implanted, it is obvious that lack of osseointegration of the femoral component could be a factor. We therefore proceeded to explore the over expression of HIF- a protein which is expressed in hypoxic conditions similar to that seen in SCD patients (chapter 3). This was done through an *in vitro* model. The conclusion from this work confirmed that the model used could mimic the *in vivo* pathologic state and also express this osteogenic protein. These results could form the basis for exploring the improvement of osseointegration. In order to give some validation to this model, we sought to explore if the osteoblasts in the model proposed did maintain some metabolic and bioviability potential despite being cultured with cobalt chloride - a hypoxic mimetic (chapter 4). It was concluded that there was still some degree of osteoblast function despite the hypoxic environment ensured with culture in cobalt chloride. We therefore suggest that this model could be further validated through more research and the results may be extrapoliated to *in vivo* models and later translated into implant design and eventually clinical practise to help improve implant survival in sickle cell patients with THR.

5.1 GENERAL DISCUSSION

One of the objectives of this research study was to propose a durable experimental model that could serve as further research platform for reducing the revision burden for hip arthroplasty in sickle cell disease patients. Dampier et al. noted in their study that despite the increase in medical advances, sickle cell disease patients still had substantial impairment of health-related quality of life in adults that are influenced by only a few of many possible medical complications. It suggests that effective treatment of persistent pain and depression would provide the largest health related quality of life (HRQOL) benefit (Dampier et al., 2011). One of the ways of addressing this in hip arthroplasty patients, is to help reduce the incidence of implant loosening and so effective osseointegration is important in achieving this. It is also important to note that there is little in the established literature that seeks to address the problem in this group of patients, hence the novelty of this experimental model, which could form a basis for addressing the problem of lack of osseointegration.

The primary experiment in this study has established the role of the hypoxic inducible factor in its being over expressed under *in vitro* hypoxic conditions. This is consistent with current literature evidence as this oxygen response system helps enhance angiogenesis and then osteogenesis, which could ultimately lead to improve prosthesis implant osseointegration. The question still needs to be asked - is there a pathologic deficiency in the over expression of HIF *in vivo* with sickle cell disease patients? Hence the lack of implant integration. Ang et al noted in their study that in a disease like familial erythrocytosis (excessive red blood cell production) could be caused by homozygosity for a hypomorphic VHL (von Hippel-Lindau syndrome) allele (Ang et al., 2002). Maxell et al therefore noted in their review, that the altered VHL function is sufficient to dysregulate erythropoiesis in humans. Presumably, this occurs by generating inappropriate signals in the fibroblasts located in the kidney and so these patients have a minor defect in HIF regulation in all cells, rather than the major defect in some cells that is seen in classical VHL disease (Maxwell, 2005). Could the genetic pathology seen here be a casual effect or similar defect in sickle cell disease patients hence the lack of implant osseointegration seen in cases with hip arthroplasty? The existing body of literature evidence is devoid of these answers and so our study helps to raise an awareness and possible experimental discuss to help answer this important question.
Orthopedic disease affects the majority of sickle cell anemia patients of which aseptic necrosis of the hip is the most common, occurring in up to 50% of patients (Vichinsky et al., 1999) and total hip arthroplasty (THA) is the long term treatment for this. There are significant functional improvements which could be achieved after THA in patients with AVN (Lee, Lee, Seol, Park, & Yoon, 2017). However it has been shown in study after study that

patients with AVN from SCD are at a higher risk during total hip replacement surgery and in the early post-operative period. Approximately a 20% or higher failure rate is expected at a mean follow-up of 10 years. The multidisciplinary approach involving haematologists, anaesthetists, and orthopaedic surgeons should be used to reduce the incidence and severity of complications (Al-Mousawi et al., 2002). The increased life expectancy that medical advances have offered to the sickle cell patient has led to the increase in sickle-cell-related complications, which were previously only seen rarely. These complications are then successfully managed (Al Elayan & Al Hamdan, 2012). This study has tried to put forward a proven hypothesis that the high failure rate seen in sickle cell disease patients with AVN treated with total hip arthroplasty can be managed. As was concluded from the second chapter which was the clinical study preamble to the experimental work, it was concluded objectively that there might be structural reasons from the shape of the proximal femur of sickle disease patients that could cause implant loosening. This conclusion also helped us to further assume from the results of other studies that the pathologic sickle cell bony environment could be a contributing factor. Hence the need to address the problem of lack of osseointegration in these patients.

The hypoxic inducible factor is an oxygen-sensitive transcriptional activator, which causes the transcriptional induction of a series of genes that participate in angiogenesis and osteogenesis (Ke & Costa, 2006). Various studies have established that osteoblasts are ideally situated in bone to sense oxygen tension and respond to hypoxia by activating the hypoxia inducible factor (HIF1α) pathway and provide evidence that HIF1α promotes angiogenesis and osteogenesis by elevating VEGF levels in osteoblasts (Rosová, Dao, Capoccia, Link, & Nolta, 2008; Wang et al., 2007). Based on this assertion, it was important to establish an experimental model to investigate the over expression of HIF in a microcellular environment which will mimic that seen in the sickle cell osteogenic pathology. In this model, the introduction of Cobalt Chloride was aimed at ensuring that true hypoxia was established *in vitro* as can be observed *in vivo* in these patients. The results from this study not only showed the up regulation of HIF as seen in documented studies but it also showed that the osteogenic protein HIF, could be augmented in the presence Cobalt Chloride. This could then play a crucial role in addressing the problem of implant loosening and improve osseointegration by the way of improved implant design.
The new generation of improved hip prosthesis could be used in sickle cell disease patients needing hip arthroplasty. However, there is some body of evidence from literature that systemic arthroprosthetic cobaltism is an increasingly recognized complication of wear or corrosion of chrome-cobalt hip implants (Cheung et al., 2016; Gessner, Steck, Woelber, & Tower, 2015). We propose from the outcome of our work that the presence of cobalt would

only be as an agent for osseointegration and not a bearing surface as seen in other implant designs.

In order to authenticate the experimental model proposed in this study, it was necessary to evaluate the cellular viability of MG63 cells cultured under the various experimental hypoxic conditions where HIF was over expressed. This was aimed at ensuring that there was osteogenic cellular viability of these cells that will help describe it as an effective and viable experimental model that could be used to investigate osseointegration. The results from the chapter four of this study, helps to establish that there was significant microcellular viability of MG63 cells after culture under 48 and 72 hours of true hypoxia. The role of cobalt chloride could be deduced from the 72 hour results, as there was no statistically significant difference in the results due to its apoptotic effects although there was increased viability as compared to the control samples. The results in this arm of the study, helps us to put forward this experimental model as novel in its use of Alamar blue assay in evaluating its role with the MG63 cells. There are few studies in the literature, which have done this.

Sickle cell disease is a genetic disorder of growing global public health importance. More than 300,000 homozygous neonates (Haemogblobin SS) suffer from sickle cell anaemia (SCA). Recent estimates based on demographic projections suggest that this number could rise to 400,000 by 2050 (Piel, Rees, & Williams, 2014; D. C. Rees, 2014). With the knowledge of the natural history of the pathology in mind, there would be an exponential increase in sickle cell patients needing hip arthroplasty surgery due to avascular necrosis hip degeneration and hence the increased revision burden. There is still paucity of research directed at answering questions about lack of implant osseointegration in these patients and this work seeks to form a foundational pathway for this with the intention of finding answers to the problem of implant loosening in these patients.

The results from this study did show first of all that the shape of the proximal femur in SCD patients when compared with that of OA patients did contribute to femoral implant loosening due to its lesser funnel shape. This along with the already sclerotic bony marrow environment does contribute to little implant osseointegration. The lower cortical thickness deduced in these patients may possibly increase risk of occult periprosthetic fractures and hence cause more loosening. The study also showed that that the microcellular bony environment of hypoxia is not a contraindication to osteogenesis and the *in vitro* model developed in this study showed the possiblity of over expression of HIF which could further enhance osseointegration in this compromised bony environment. The results noted from this model was then further verified by establishing that osetoblasts cultured in hypoxia, did

have functional potential and viabilty and so increasing its prospects for its future use *in vivo.* The experimental model developed here could be modified to further help design further models which could investigate osseointegration or the lack of it in sickle cell disease patients *in vivo.* With the further possibilty of HIF being over expressed in hypoxic conditions as shown in this model, there is an obvious futuristic potential of this osteogenic protein being used as a coating on femoral implants implanted in SCD patients in order to enhance osseointegration. Therefore more research work needs to be aimed at achieving this goal as it would go along way in improving implant survival rate in these patients.

CHAPTER SIX- CONCLUSIONS AND FUTURE WORK

6.0 CONCLUSIONS

Hip arthroplasty implant failure in sickle cell disease patients continues to impact on the quality of life in these patients. With the increase in life expectancy in this group of patients, the surgical revision burden would exponentially increase, more so that most of these patients are young and active. The cost of revision is known to be signifcantly higher than the primary hip procedure in these cases. It is therefore imperative that directed research is undertaken to help improve implant osseontegration in these patients which would lead to improved survival rates. This would invariably in no small measure reduce significantly the implant revision burden. This work ultimately sought to answer some questions about the lack of implant osseointegration.

The clinical study carried out through radiographic evaluation highlighted that the shape of the proximal femur in sickle cell disease patients may be a causative factor in the failure of femoral component of the hip prosthesis. It was established that the proximal femur in these patients was funnel shaped allowing for some stable press fit implant fixation. However OA patients exhibited more funnel shaped femur and thicker cortices. This could represent increased risk of periprosthetic fracture in SC patients and eventually leading to less stable implant fixation. This may also led to the assumption that the lack of osseointegration could be the overarching factor in implant failure in these patients.

An experimental model was then designed to help assess the expression of the hypoxic inducible factor (HIF) an osteogenic protein in hypoxic conditions. This model sought to mimic the *in vivo* hypoxic pathologic bone environment seen in sickle cell disease patients. Here cobalt chloride was used as a hypoxic mimetic agent and the cultured bone was assessed for its physiologic viability. The results from this arm of the study did show that HIF over expressed was a reality and bone cultured here had significant functional potential. The experimental model used here could be instrumental in forming the foundations for further *in vivo* studies directed at investigating and improving implant osseointegration in these group of patients. Cobalt could be an agent for enhancing osseointegration as shown here in this model in contrast to its use as a bearing sufrace in current implant technology. It is obvious that improved prosthesis design aimed at ensuring effective bone implant integration could use the template in this study as the basis for novel treatment of titanium surfaces and thus enhance bone-implant bonding which will invariably improve the quality of life of sickle cell disease patients with hip replacements.

6.1 FUTURE RESEARCH PROPOSALS

6.1.1 Scope of Problem

There has been several experimental and clinical studies aimed at finding the ideal hip arthroplasty modality in sickle cell disease patients (Celebi et al., 2006; Gulati et al., 2015; Jack et al., 2016). The jury is still out on whether cemented or cementless implants give the best results. Some authors have suggested that due to lack of implant integration in these patients, cemented implants should be used. However others have speculated that cement, with its thermal effect, causes further necrosis of the already poorly vascularised bone and the necrosed bone may provide a better environment for the activation and growth of the dormant bacteria which are frequently cultured from the sickler's bone (Sanjay & Moreau, 1996). It has also been suggested in this study previously that the proximal femur of SCD patients is funnel shaped and hence allowing for press fit prosthetic implantation to occur in these patients during hip arthroplasty procedures. Although it has been well documented that there are well founded surgical technical difficulties (Clarke et al., 1989; Platt, Rosenstock, & Espeland, 1984). Some studies with encouraging outcomes have been done looking at the results from cementless implants for osteonecrosis patients (Chiu, Shen, Ko, & Chan, 1997; Hartley, McAuley, Culpepper, & Engh, 2000). However, available literature is still devoid of large numbered studies with significantly long term follow up in this group of patients in order to discard doubts and controversies currently in the orthopaedic surgical community. This lack of clarity further reinforces the need for more integrated research work aimed at addressing the question of implant loosening. Some of the suggestions for further research based on the results from this work are highlighted below. Furthermore there has been good and effective advances in the overall management of sickle cell anaemia through preventative and curative approaches to therapy (Ballas, 2002) and this has seen the life expectancy in these patients increase. It is therefore desirable that research is also directed at improving their quality of life through more enhanced osseointegration, thereby reducing significantly the high revision rate.

6.2 *Prospects for Improved Implant Design*

Hip implant design is crucial in addressing the problem of lack of osseointegration in sickle cell disease patients. Several studies in the literature have highlighted the use and efficacy of osteogenic-coated femoral stems in total hip arthroplasty. Many orthopaedic surgeons consider the use of hydroxyapatite (HA) for the potential advantages of increasing the strength of the implant-to-host bone bond and decreasing the amount of time required to achieve stable fixation (Cook et al., 1992; Soballe et al., 1991). Furthermore Sanz-Reig et al. showed in their studies that 98% of titanium plasma sprayed implant stems used in THA had signs of stability (endosteal bone formation and proximal adaptive bone remodeling) and 61% had endosteal spot welds indicative of bony fixation (Sanz-Reig, Lizaur-Utrilla, Llamas-Merino, & Lopez-Prats, 2011). *In vitro* and *in vivo* studies have shown the efficacy of osteogenic substrates such as human bone marrow stem cell seeded on poly (dl-lactic acid) scaffolds as potential biologic bone graft extended for future use in bone grafting (Bolland et al., 2008). It is also worth noting that other studies including randomized controlled trials have highlighted the lack of significant difference in clinical advantage for the use of HA coated implants (Camazzola, Hammond, Gandhi, & Davey, 2009). The reason for the disparity in the literature could be the need to further explore effective osseointegration in the design of hip implants.

The results gleaned from this work showed that HIF, which is a hypoxic response protein, could be over-expressed in the microcellular hypoxic environment in the presence of cobalt chloride - a mimetic for hypoxia. It was also established that cellular viability was present even in this hypoxic environment, which was modelled after the osteopathologic environment seen in osteonecrosis – a component in the natural history in sickle cell bony disease. Knowing the complication of implant loosening in these patients, could HIF conjugated unto a known polysaccharide and seeded as a coating unto titanium hip implants be used to ensure effective osseointegration? A lot of work has been done in improving the titanium implant surface in order to enhance bony implant integration. Della et al suggested in their work that silicon based anodic spark deposition treatment of the titanium surface would enhance osseointegration in orthopaedic applications (Della Valle, Rondelli, Cigada, Bianchi, & Chiesa, 2013). In another study, they established that the modification treatment of the titanium surface could confer antibacterial properties, which could ensure osseointegration and prevent septic loosening (Della Valle et al., 2012). Sandrini et al also noted in their study that the novel biomimetic treatment of the titanium surface, led to faster and durable implant to bone bonding through higher mineralization capacity (Sandrini, Chiesa, Rondelli, Santin, & Cigada, 2003). Having noted the above studies as references, our study could be used as

an experimental model to help improve the titanium surface osteogenic potential by treating it with HIF conjugated composite seeded on the implant. Like most of the studies referenced, this study is an *in vitro* model, which needs to be tested *in vivo* in order to establish its efficacy as a clinical application in sickle cell patients. Implant design research aimed at improved osseointegration could benefit from these proposals and the result would inevitably be a reduction in the revision burden and high implant failure rate observed in these patients.

RESEARCH RELATED PRESENTATIONS/PUBLICATIONS

PUBLICATIONS

1. Accepted for Publication in EFORT OPEN REVIEWS JOURNAL - The Hypoxic Inducible Factor (HIF): How to Improve Osseointegration in Hip Arthroplasty Secondary to Avascular Necrosis in Sickle Cell Disease- September 2018

ORAL PRESENTATIONS

1. George A.O., Ellis M.J.E, Gill H.S- The Radiographic Evaluation of the Proximal Femur in Sickle Cell Disease Patients with Avascular Necrosis. *The European Federation of Orthopaedic Trauma Scientific Congress. Geneva, Switzerland. May 2016 (Poster Walk -oral presentation)*

2. George A.O., Ellis M.J., Gill H.S- The Radiographic Evaluation of the Proximal Femur in Sickle Cell Disease Patients. *The European Orthopaedic Research Society. 25th Annual Meeting. Munich, Germany. September 2017. (Podium presentation).*

3. George A.O., Ellis M.J., Gill H.S. The Radiographic Evaluation of the Proximal Femur in Sickle Cell Disease Patients with Avascular Necrosis. *The West African College of Surgeons 58th Annual Conference and Scientific Meeting. Banjul, Gambia. February 2018. (Podium presentation)*

4. George A.O., Ellis M.J., Gill H.S. The Effect of Cobalt Chloride on Human MG63 Osteoblast Cellular Metabolism-Using an *In Vitro* Experimental Model. *The European Federation of Orthopaedic Trauma Scientific Congress. Barcelona, Spain. May 2018*

5. George A.O., Ellis M.J., Gill H.S. An Experimental Study to Investigate a Potential Model for Improved Osseointegration in Sickle Cell Bone Disease Patients with Avascular Necrosis. *The European Orthopaedic Research Society. 25th Annual Meeting. Galway, Ireland. September 2018. (Podium presentation).*

6. George A.O., Ellis M.J., Gill H.S. Experimental Study to Investigate a Potential Model for Improved Osseointegration in Sickle Cell Bone Disease Patients with Avascular Necrosis. *The 13th Congress of the European Hip Society. The Hague, Netherlands. September 2018*

APPENDIX

FIGURE A1-*Anterioposterior radiography of a 27 hear old female sickle cell patient with right femoral head avascular necrosis.*

INTEROBSERVER RELIABILITY DATA FOR SICKLE CELL GROUP (Tables A1 – A3)

Table A1 Inter-item correlation Matrix for X (mid-diaphysis canal measurements). *Intra-class correlation excellent amongst observer measurements (p value <0.001).*

	X1	X2	X3
X1	1.000	0.992	0.997
X2	0.992	1.000	0.995
X3	0.997	0.995	1.000

Table A2 Inter-Item Correlation Matrix for Y (proximal femoral canal measurements).
Intra-class correlation excellent amongst observer measurements (p value<0.001)

	Y1	Y2	Y3
Y1	1.000	0.971	0.987
Y2	0.971	1.000	0.985
Y3	0.987	0.985	1.000

Table A3 Inter-Item Correlation Matrix for Z- extra medullary diaphysis measurement.
Intra-class correlation excellent amongst observer measurements (p value<0.001)

1- Observer one: 2-observer two: 3 - observer three

	Z1	Z2	Z3
Z1	1.000	.991	0.992
Z2	0.991	1.000	0.999
Z3	0.992	0.999	1.000

INTEROBSERVER RELIABILITY DATA FOR OSTEOARTHRITIC GROUP (Tables A4-A6)

Table A4 Inter-Item Correlation Matrix for x (mid-diaphysis canal measurements) O.A group. *Intra-class correlation excellent amongst observer measurements (p value<0.001)*

	x1	x2	x3
x1	1.000	0.907	0.478
x2	0.907	1.000	0.472
x3	0.478	0.472	1.000

Table A5 Inter-Item Correlation Matrix for y (proximal femoral canal measurements). *Intra-class correlation excellent amongst observer measurements (p value<0.001)*

	y1	y2	y3
y1	1.000	.889	0.870
y2	0.889	1.000	0.741
y3	0.870	0.741	1.000

Table A6 Inter-Item Correlation Matrix for z-extra medullary diaphysis measurement. *Intra-class correlation excellent amongst observer measurements (p value<0.001)*

1-observer one: 2-observer two: 3-observer three

	z1	z2	z3
z1	1.000	0.461	0.531
z2	0.461	1.000	0.906
z3	0.531	0.906	1.000

MICROSCOPIC IMAGES OF CELL CULTURE SAMPLES UNDER CONTROL (NORMOXIA) AND EXPERIMENTAL (HYPOXIC) CONDITIONS AT 48 HOURS AND 72 HOURS WITH AND WITHOUT CHLORIDE

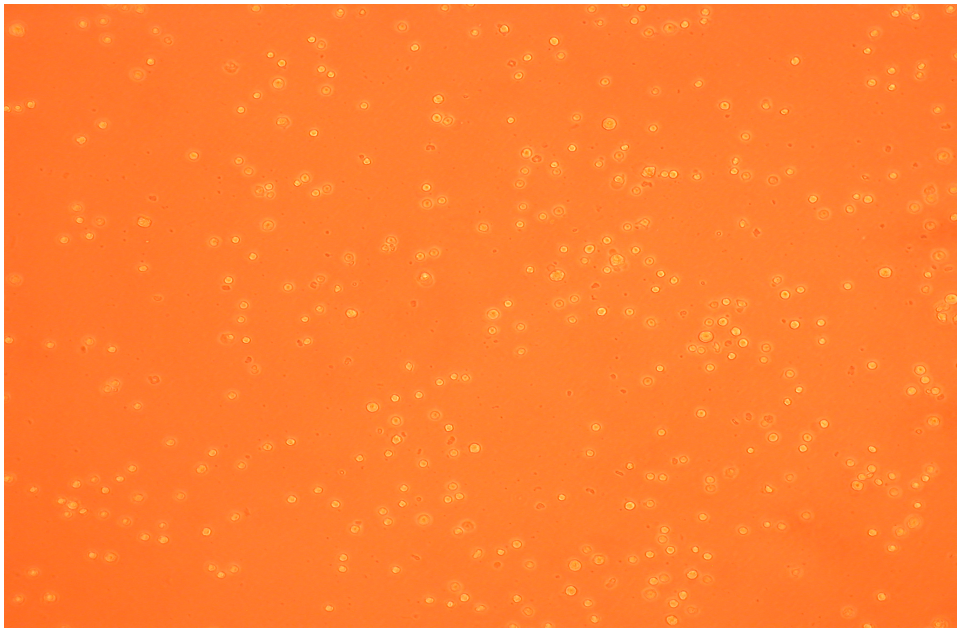

Figure A2-*MG63 cells cultured under 20% normoxia with cobalt chloride for 48hours (40x-magnification)*

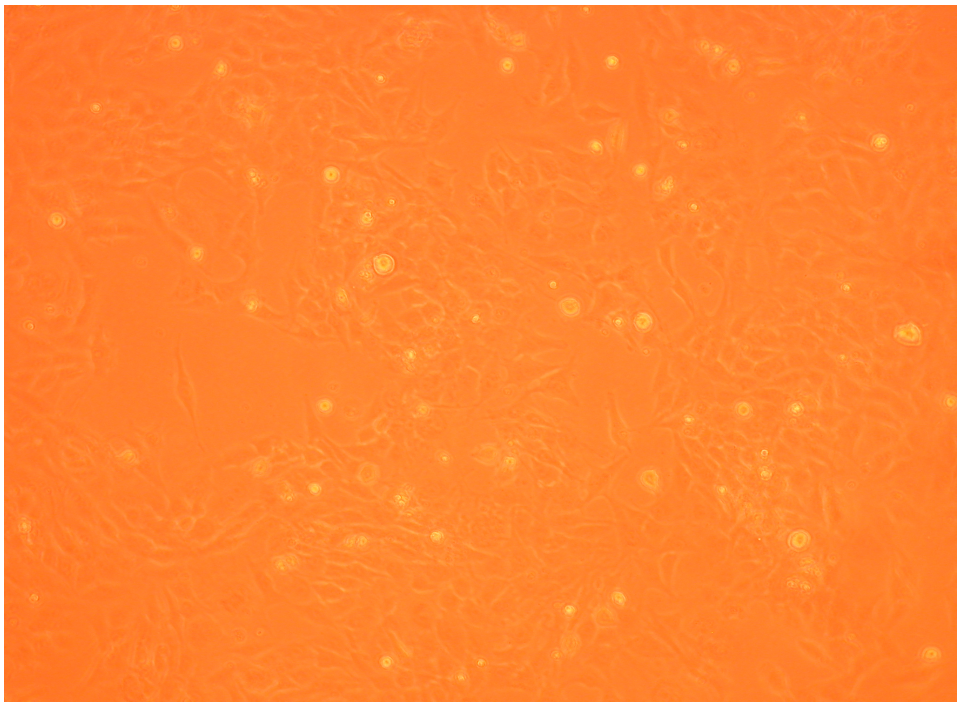

Figure A3- *MG63 cells cultured under 20% normoxia without cobalt chloride for 48hours (40x-magnification)*

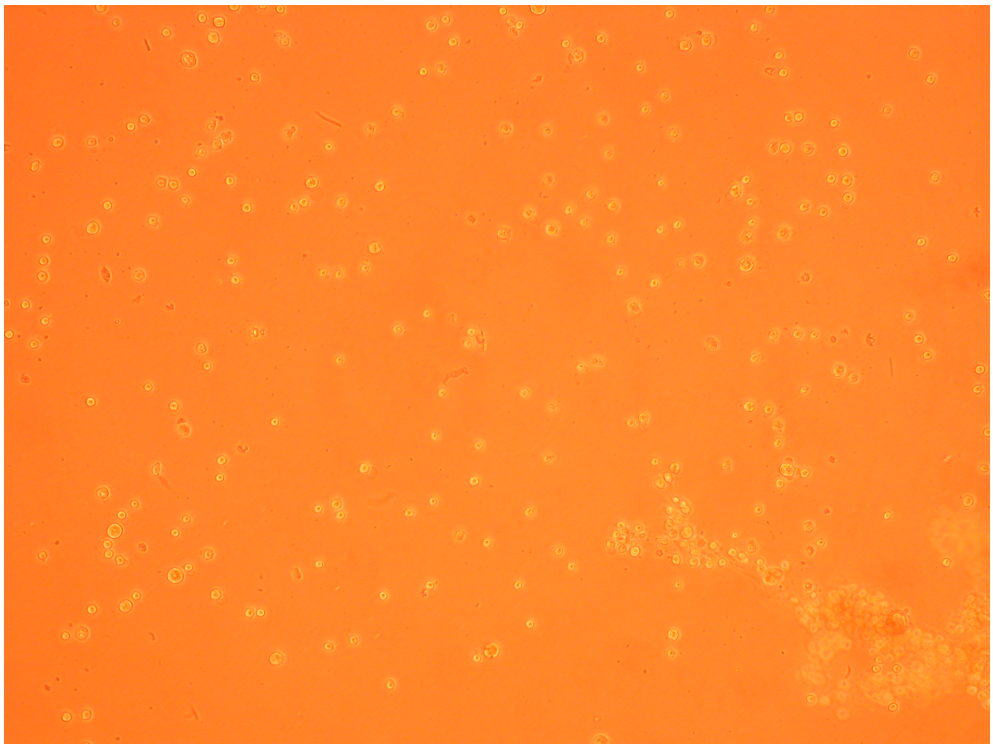

Figure A4 - *MG63 cells cultured under 1% hypoxia with cobalt chloride for 48hours (40x-magnification)*

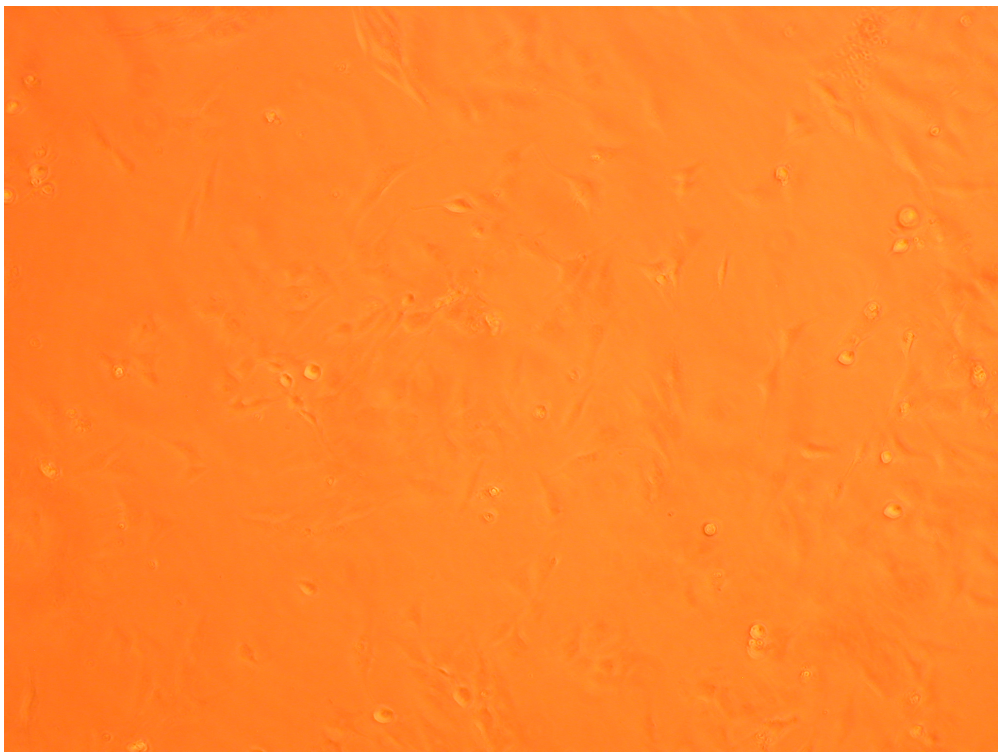

Figure A5 - *MG63 cells cultured under 1% hypoxia without cobalt chloride for 48 hours* ***(40x-magnification)***

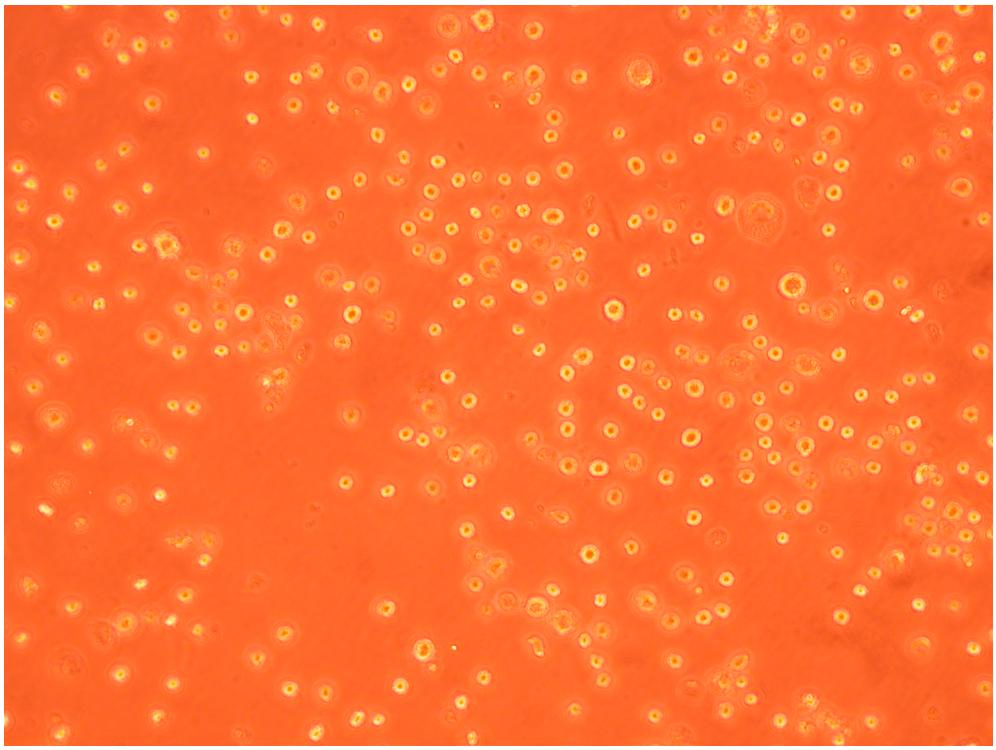

Figure A6- *MG63 cells cultured under 20% normoxia with cobalt chloride for 72hours (40x-magnification)*

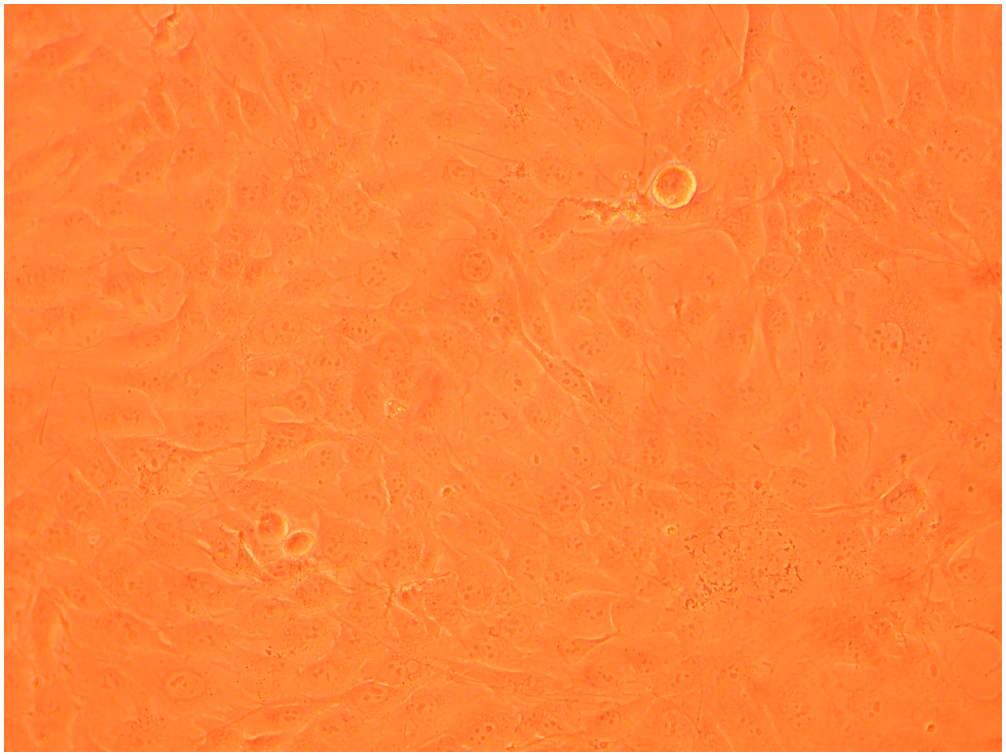

Figure A7- *MG63 cells cultured under 20% normoxia without cobalt chloride for 72hours (40x-magnification)*

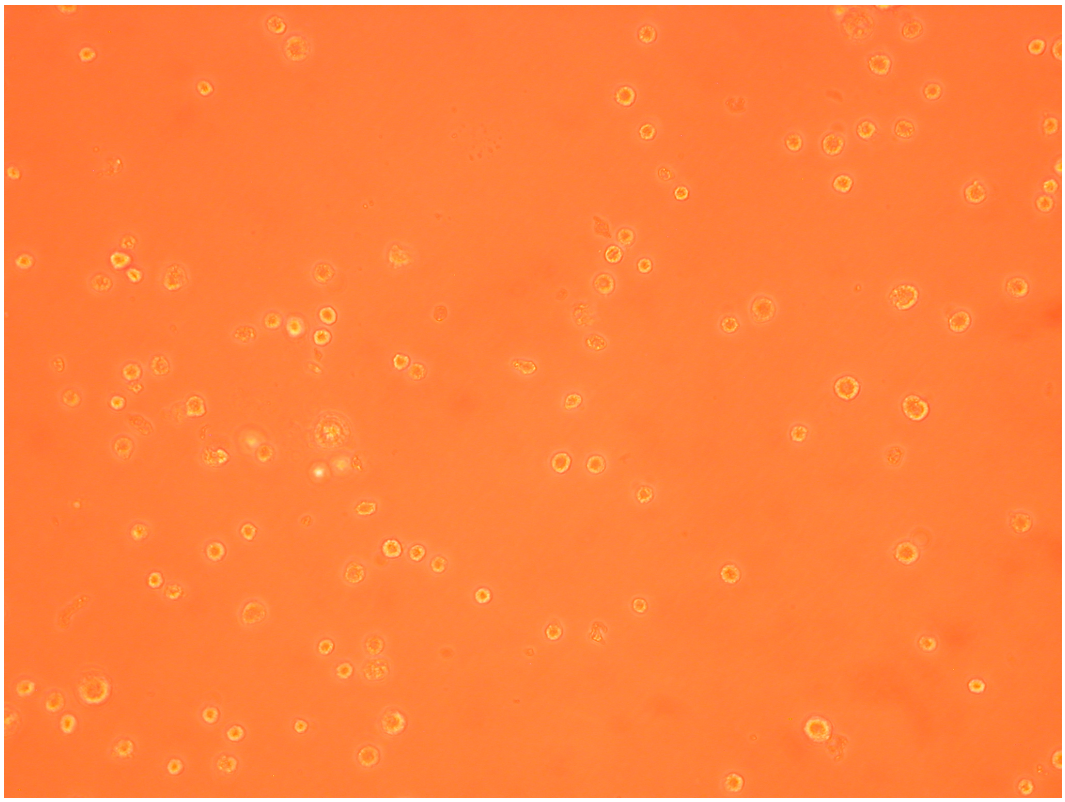

Figure A8- *MG63 cells cultured under 1% hypoxia with cobalt chloride for 72 hours (40x-magnification)*

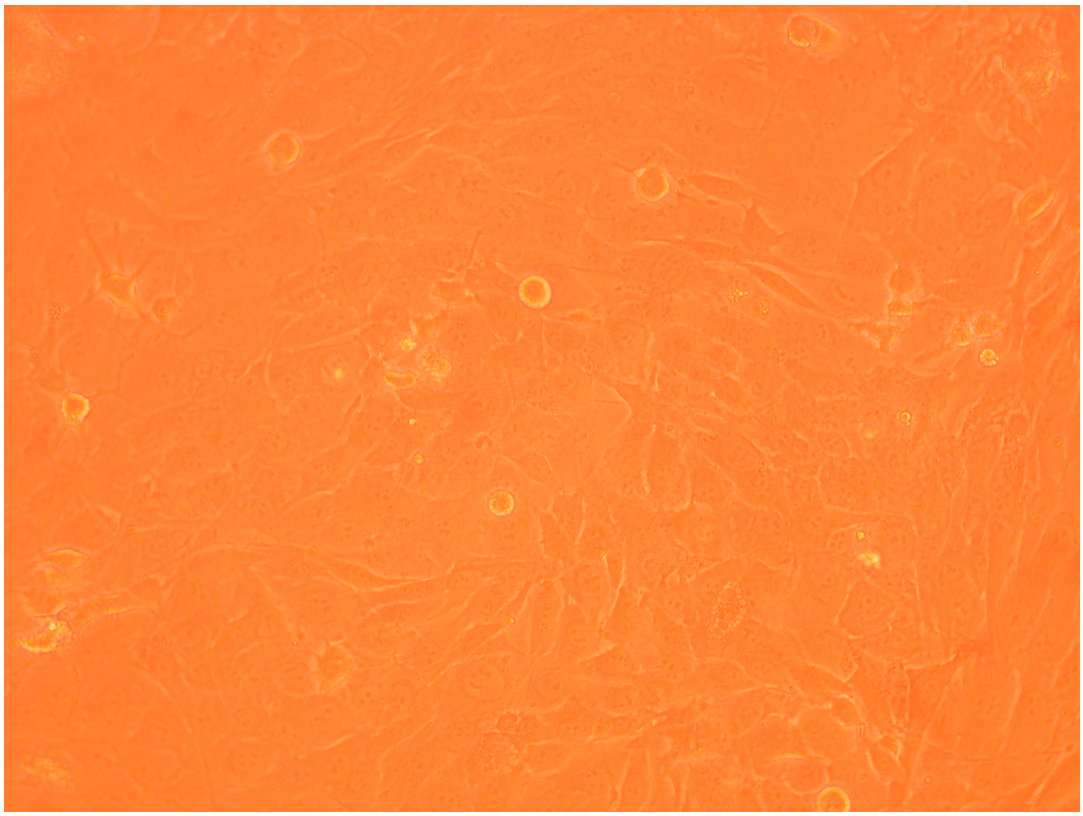

FIGURE A9- MG63 cells cultured under 1% hypoxia without cobalt chloride for 72hours

(40x-magnification)

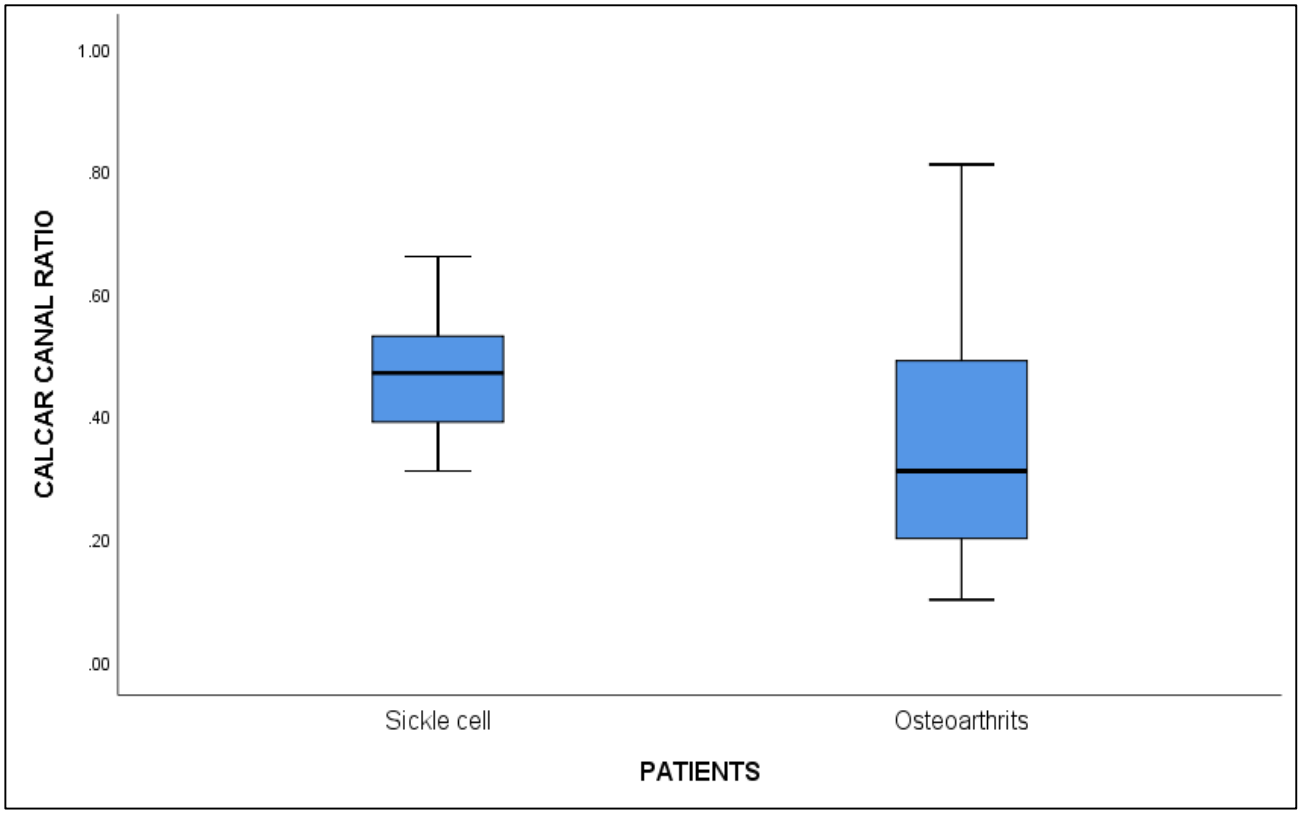

Figure A10- *Showing box plot for calcar canal ratio data with no outliers.*

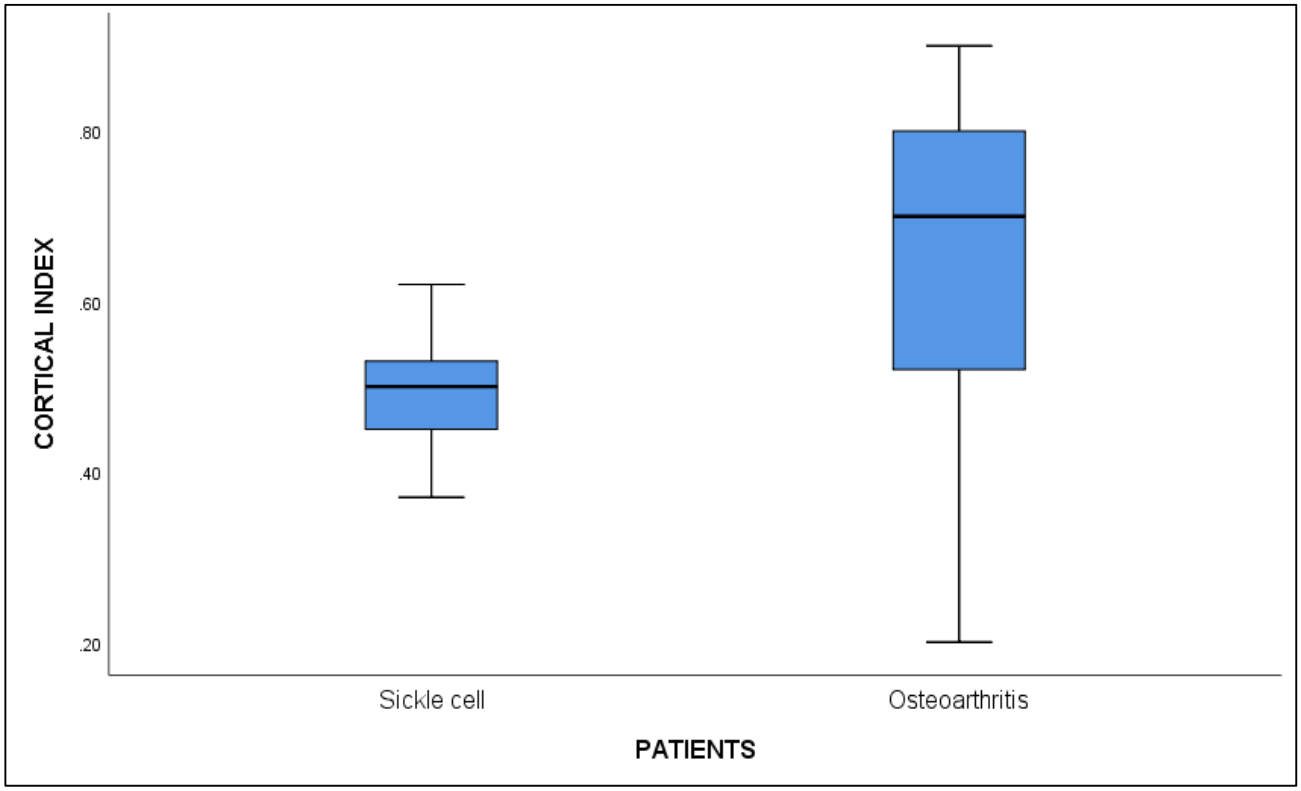

Figure A11- *Showing box plot for cortical index data with no outliers.*

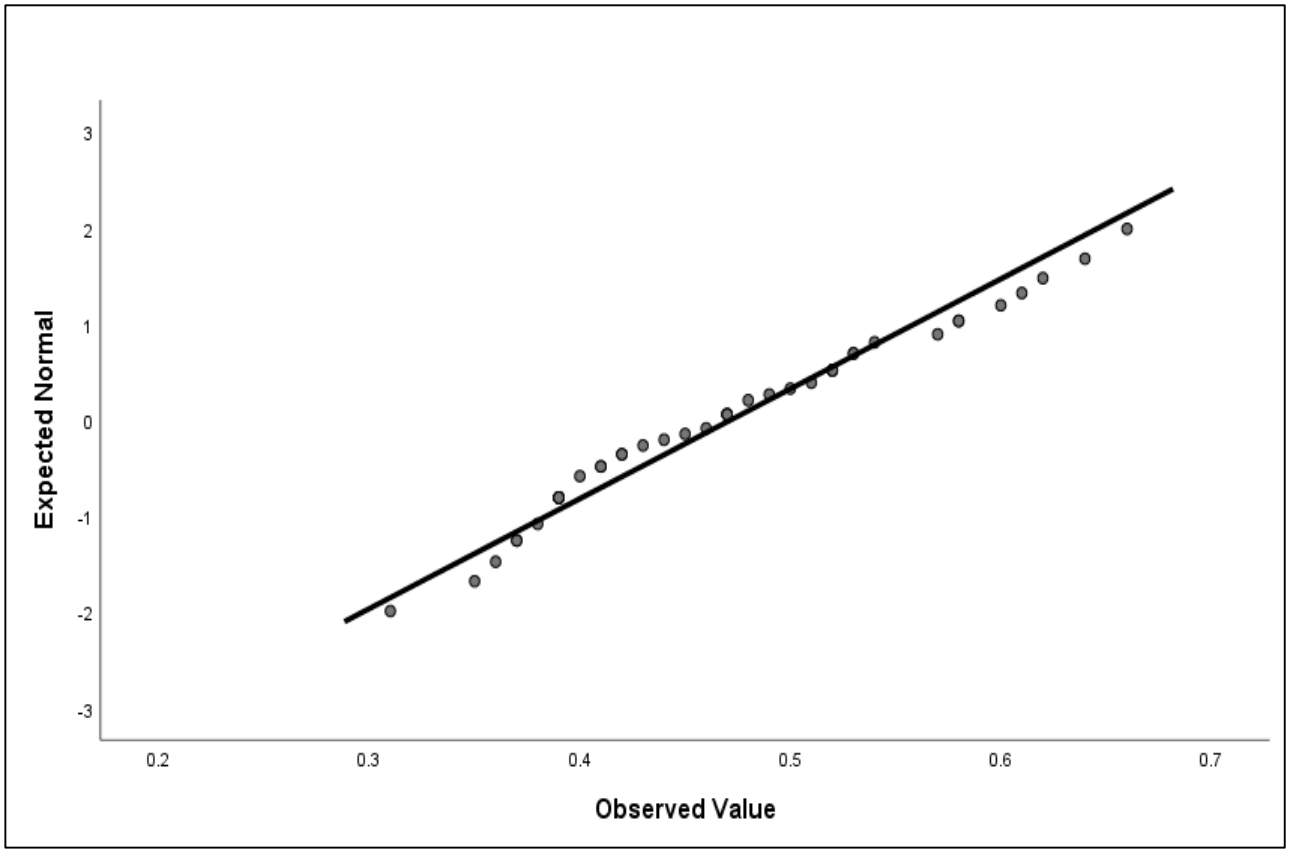

Figure A12- *Showing Normal Q-Q plot of Calcar Canal ratio for Sickle Cell patients confirming normality of data.*

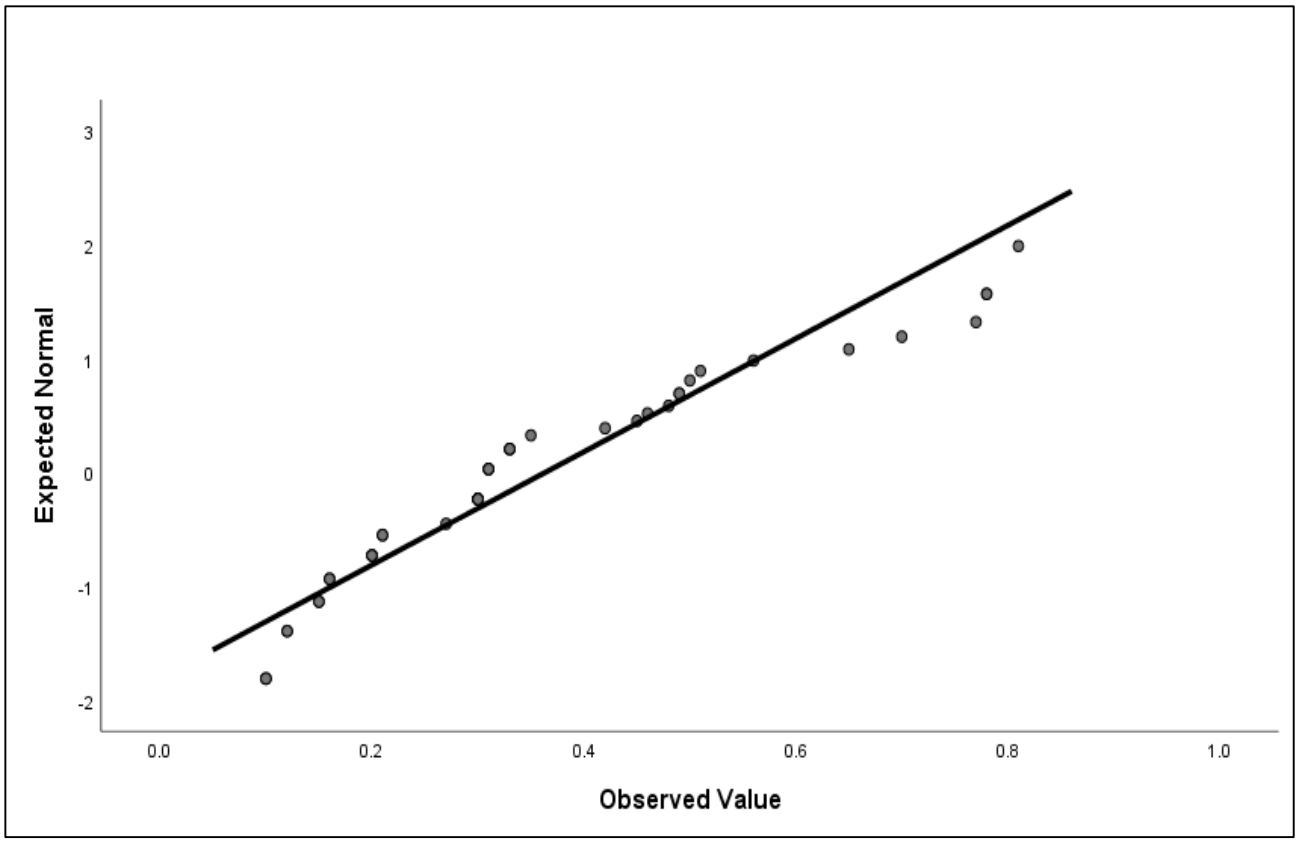

Figure A13- *Showing Normal Q-Q plot of Calcar canal ration for Osteoarthritis patients confirming normality of data.*

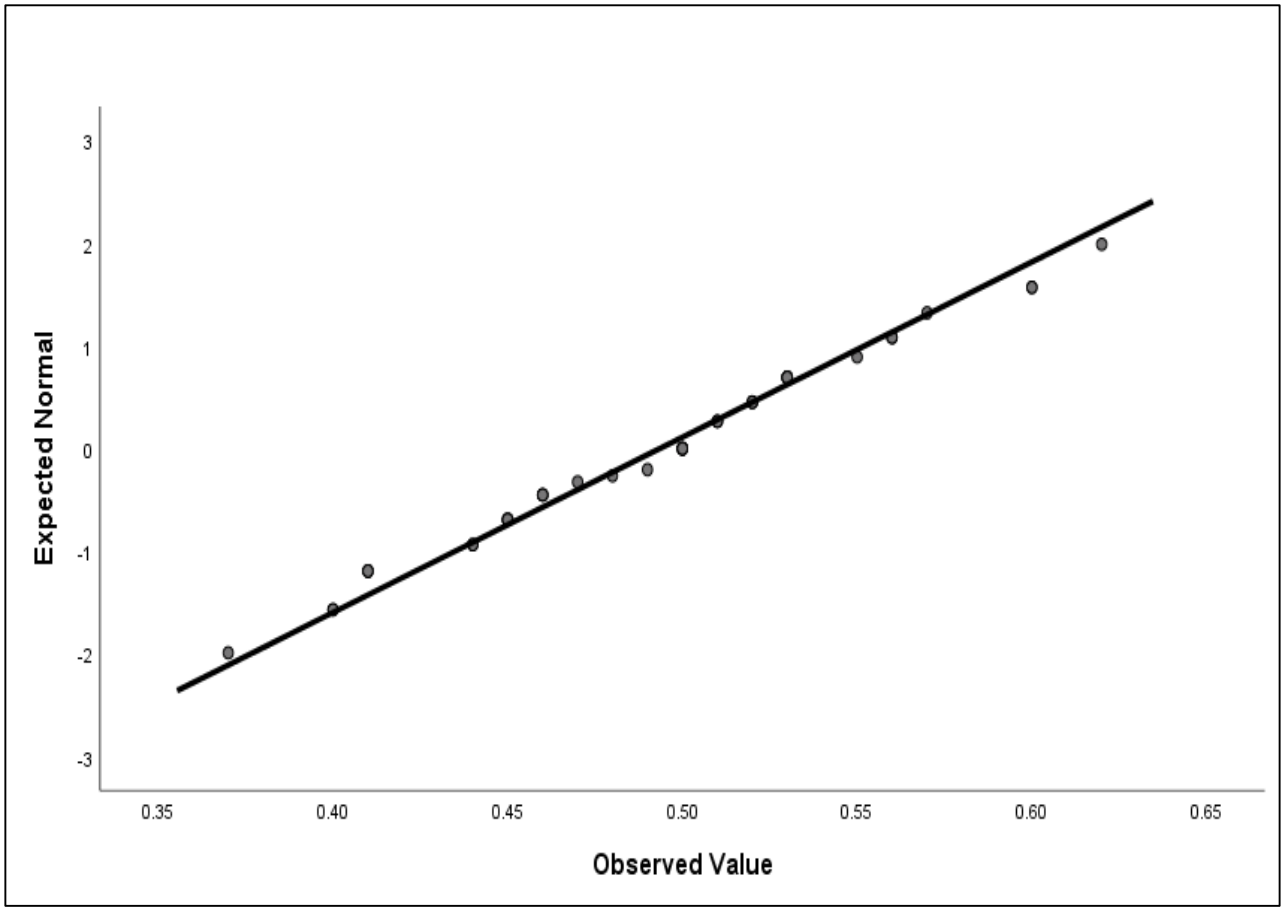

Figure A14 - *Showing Normal Q-Q PLOT of Cortical Index (CI) for Sickle Cell patients confirming normality of data.*

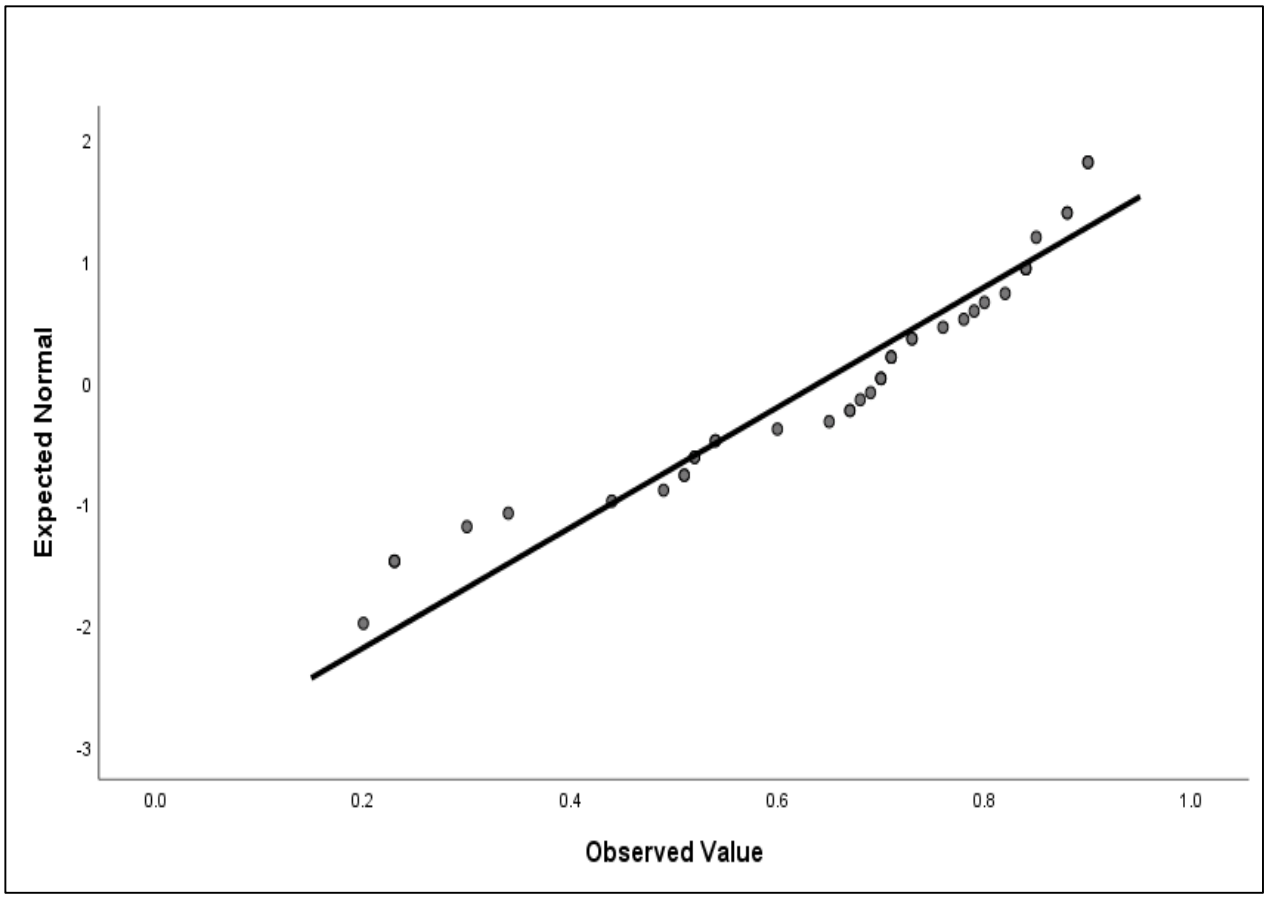

Figure A15 – *Showing Normal Q-Q Plot for Cortical Index for Osteoarthritic patients- confirming normality of data.*

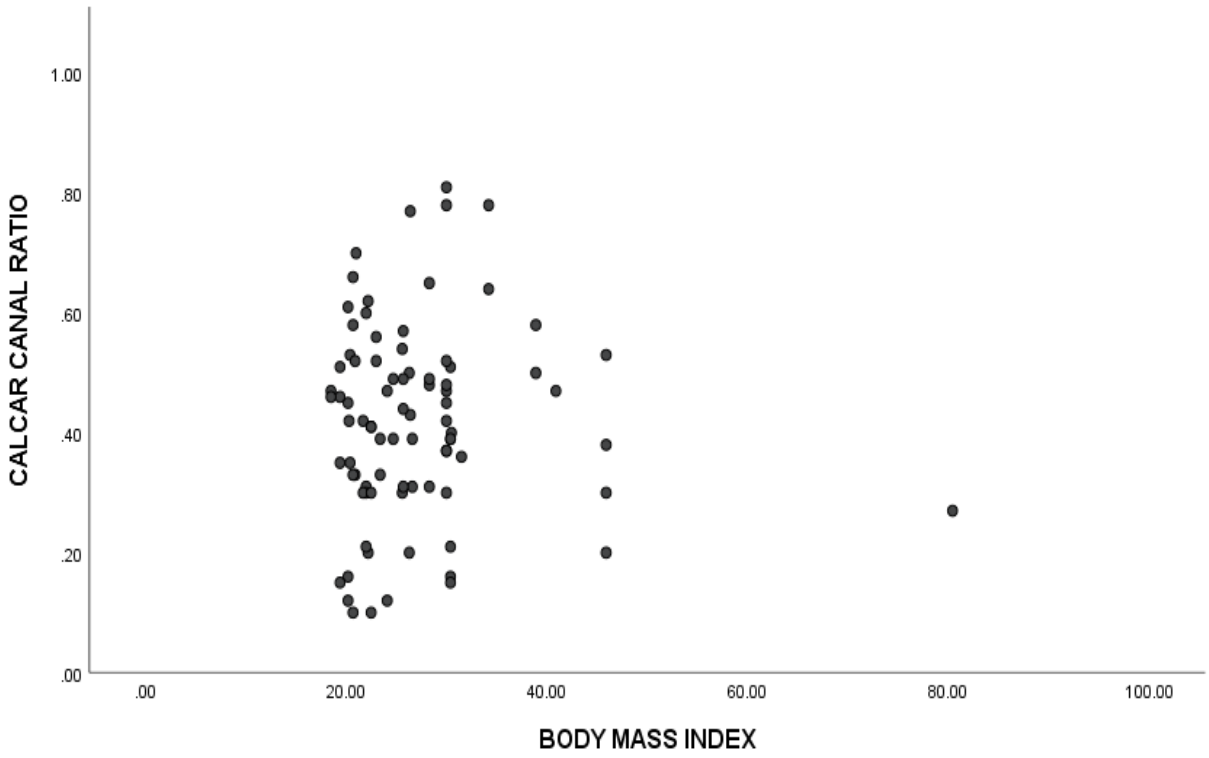

Figure A16- *Showing Scatter plot graph of Calcar Canal ratio versus Body Mass Index- Indicating a non-monotonic relationship.*

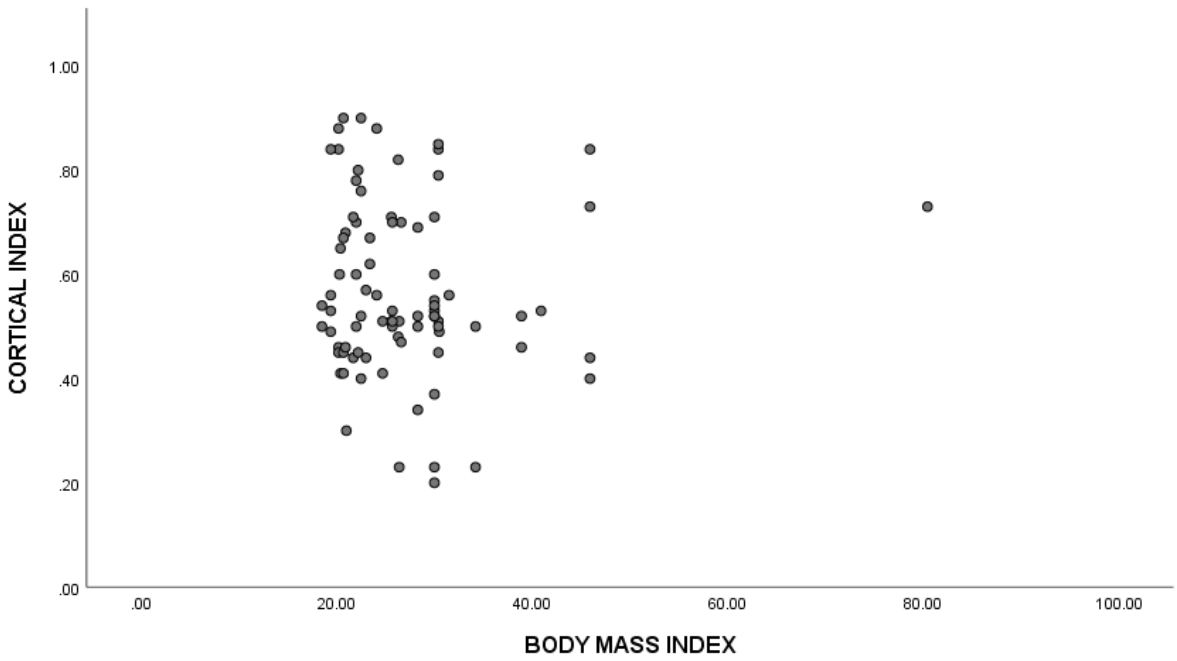

Figure A17- *Showing Scatter plot graph of Cortical Index versus Body Mass Index- Indicating a non-monotonic relationship.*

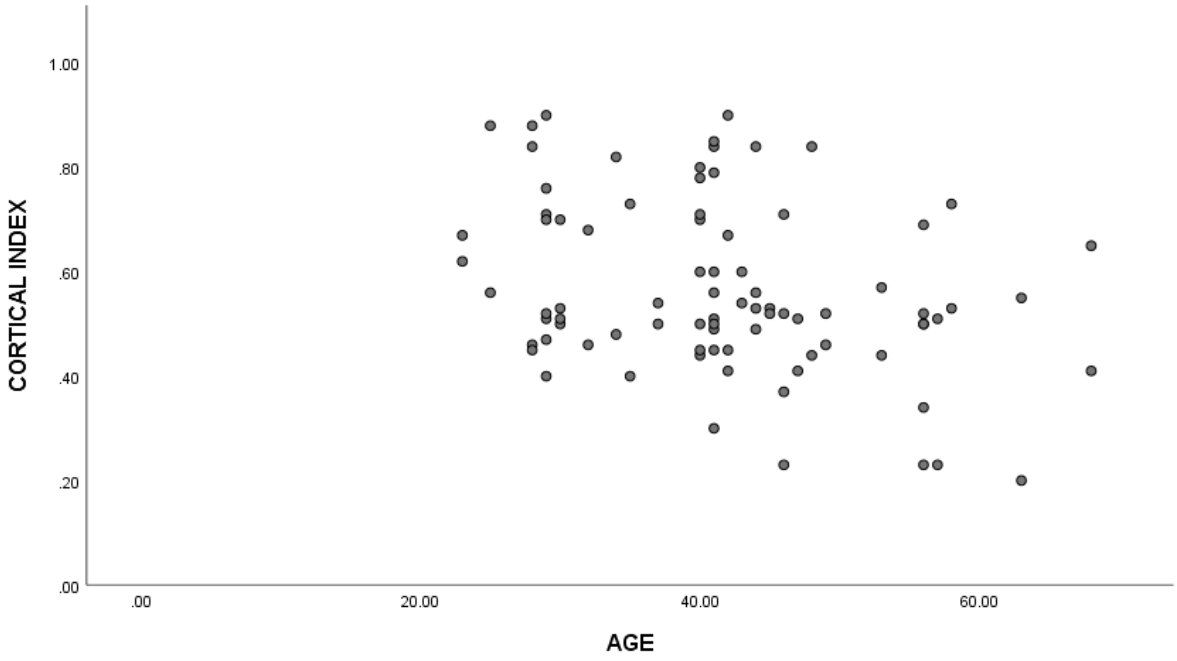

Figure A18- *Showing Scatter plot graph of Cortical Index versus Age, Indicating a monotonic relationship*

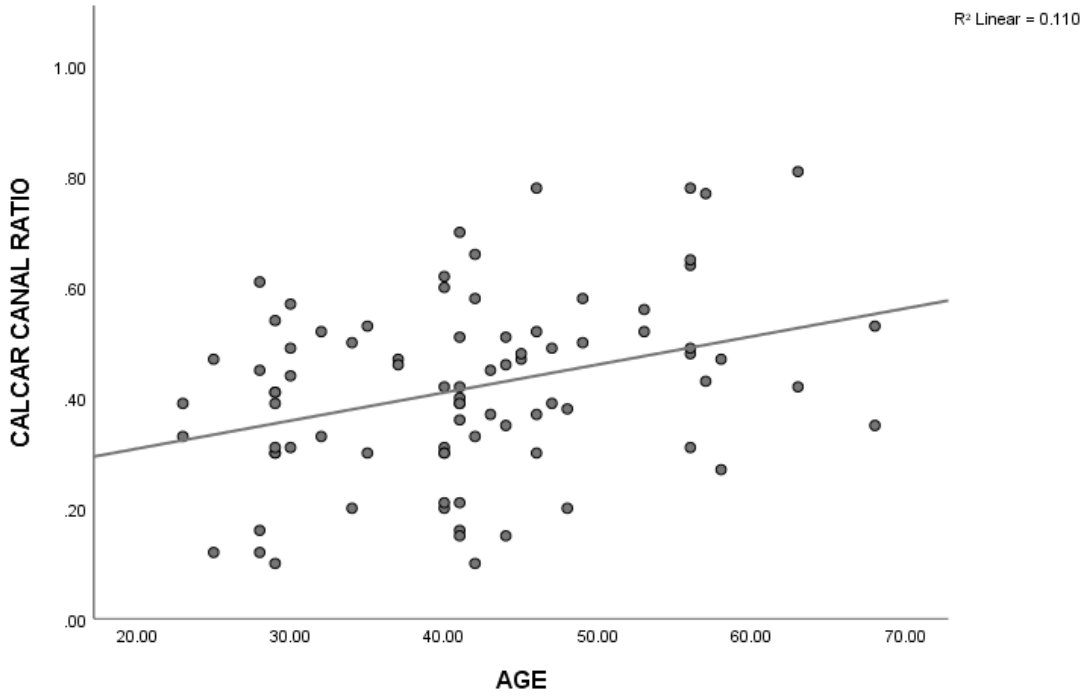

Figure A19- *Showing Scatter plot graph with fit line of Calcar canal Ratio versus Age- indicating a linear relationship.*

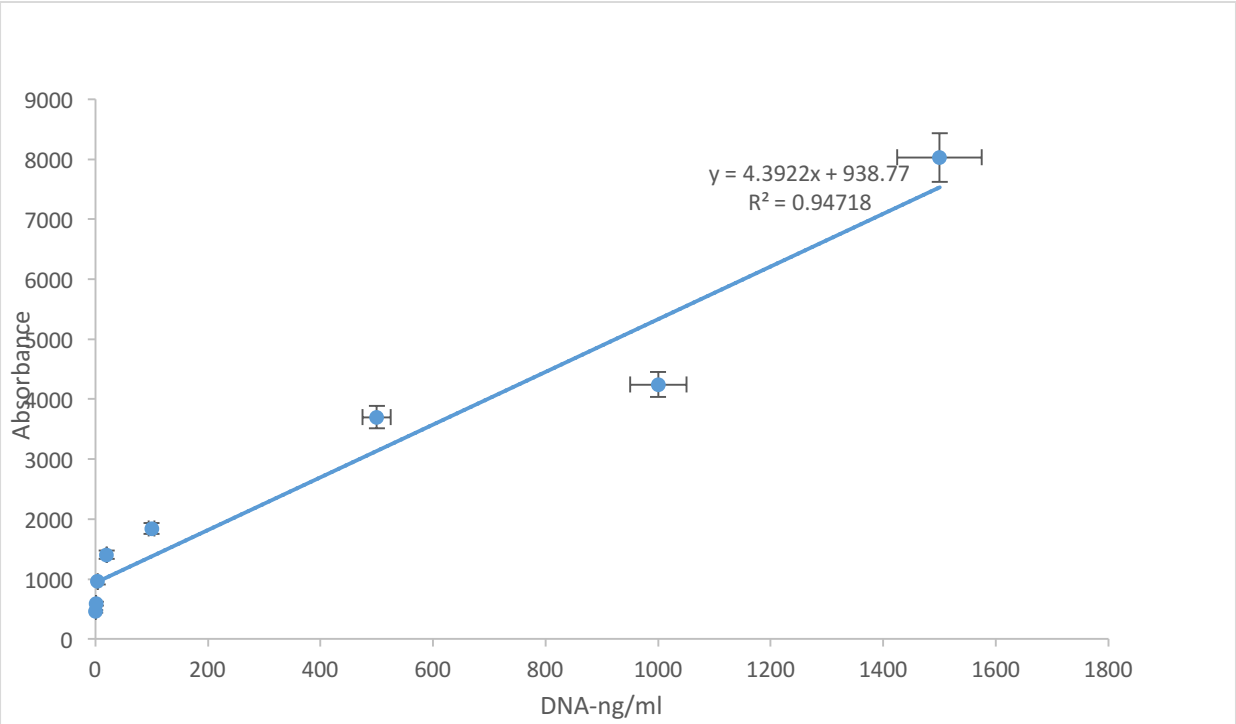

FIGURE A20- *DNA standard curve for Picogreen Assay showing Absorbance versus DNA cell concentration (n=3:N=3).*

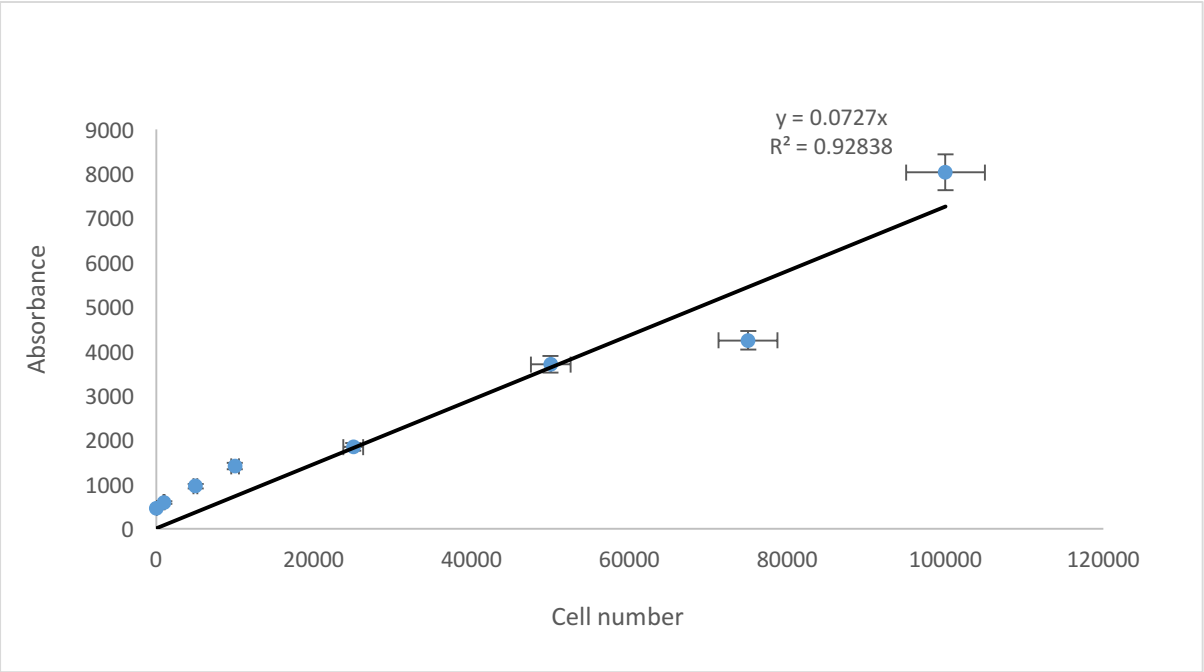

Figure A21- *MG63 Cell number standard curve for picogreen assay showing Absorbance versus Cell number (n=3:N=3).*

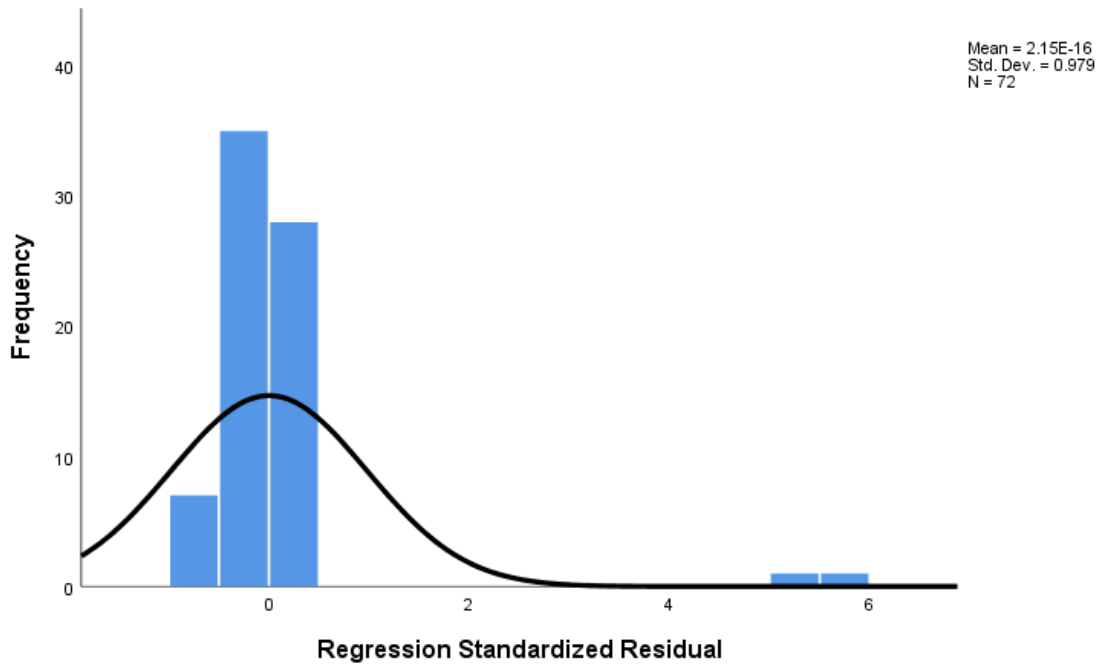

Figure A22-*Showing histogram showing standardized residuals for the absorbance per cell- showing normal distribution.*

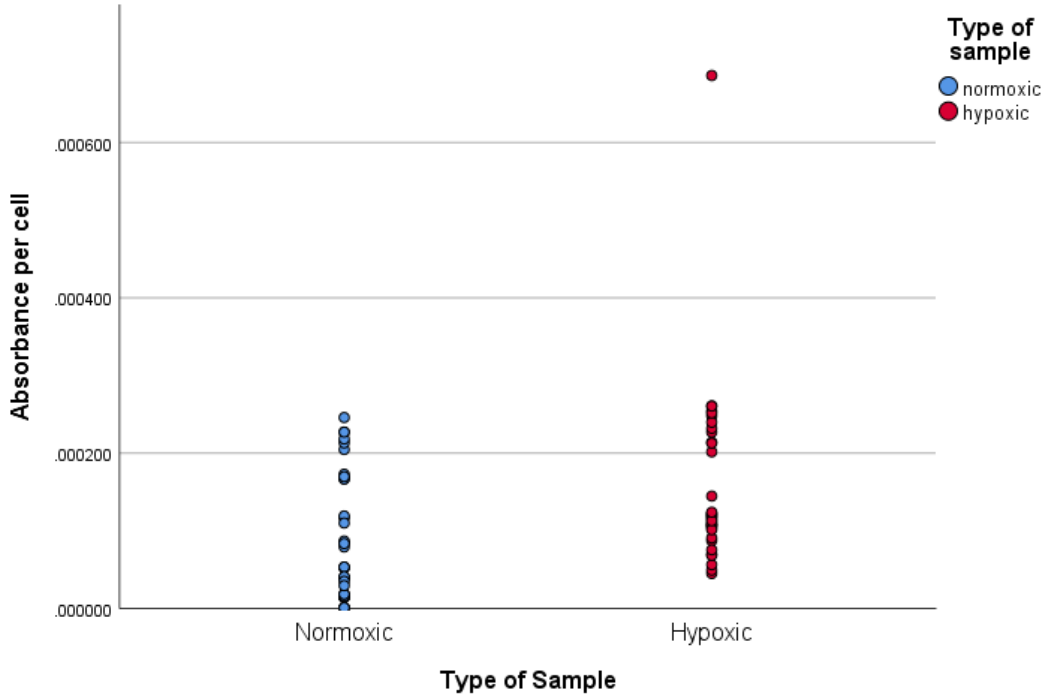

Figure A23- *Showing grouped scattered plot of absorbance per cell by type of sample, confirming homoscedasticity of data.*

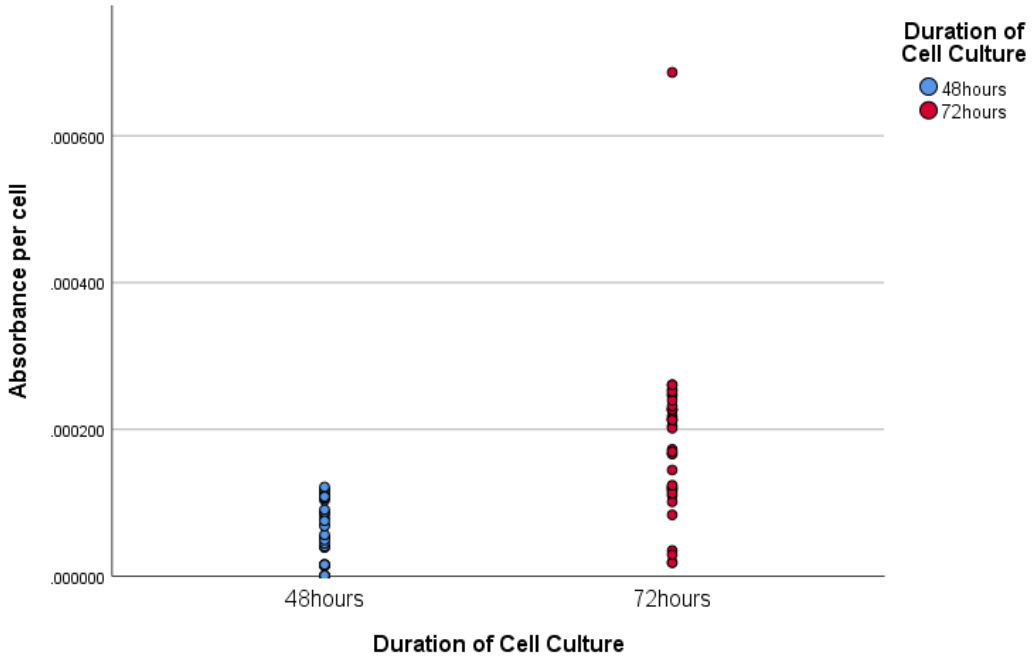

Figure A24- *Showing grouped scattered plot of absorbance per cell by duration of sample, confirming homoscedasticity of data.*

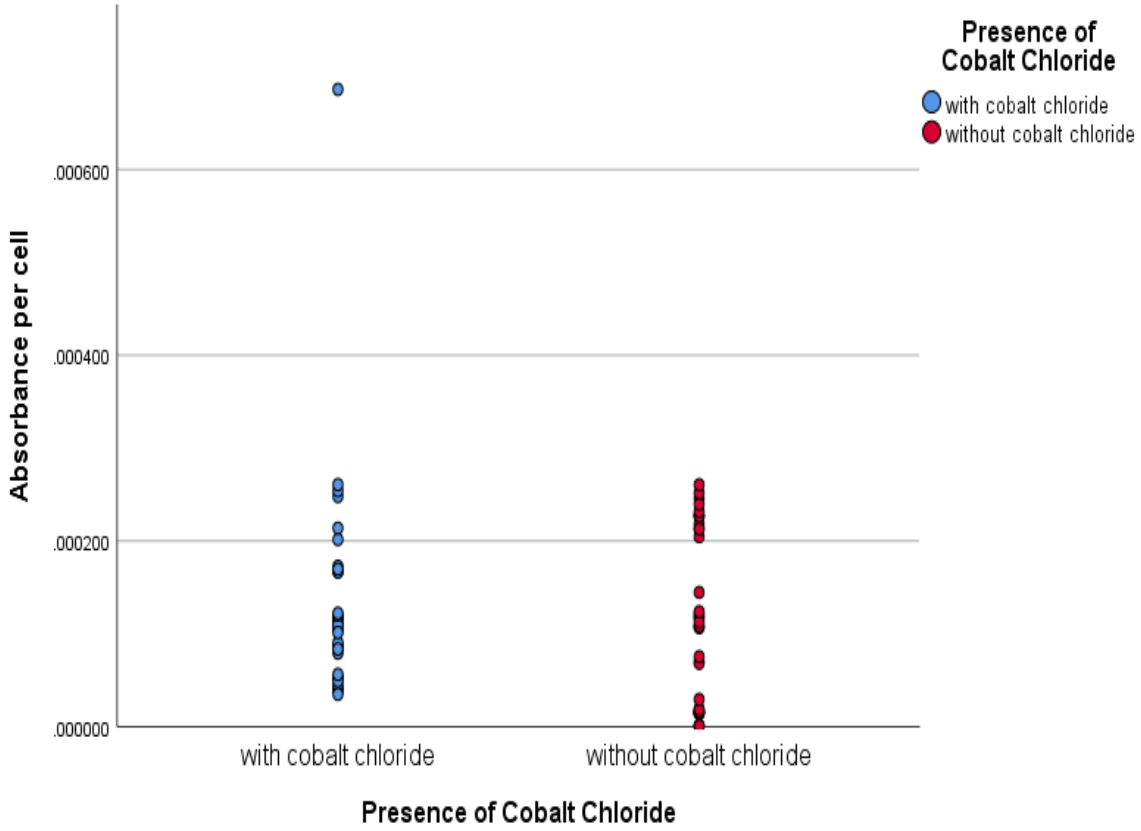

Figure A25- *Showing grouped scattered plot of absorbance per cell by presence of Cobalt Chloride in sample, confirming homoscedasticity of data.*

PHYSICAL CHARACTERIZATION EXPERIMENT FOR THE FOR-GALAXY R-O2 OXYGEN INCUBATOR-*(Model No. 170-300)*

AIM

The objective of this experiment was to assess the baseline oxygen saturation reading of the incubator comparing the set measurements taken with the observed. The reason for this is to ascertain if the experimental conditions, which are 1% oxygen saturation- hypoxia under which cell samples were cultured, are stable and reproducible.

MATERIALS AND METHODS

The Galaxy R-oxygen incubator ((Model No. 170-300) - was connected to the nitrogen cylinder. The incubator oxygen sensor automatically calibrates to atmospheric levels. According to the incubators, instructional manual the incubator was set to 1% and the nitrogen released into the chamber. The incubator has previously been connected to the computer and oxygen pressure readings was taken. The chamber readings were noted for 24 hours.

RESULTS.

The readings for the set and observed partial pressure of oxygen in the incubator chamber shows that a similar trend of readings *(See Figures 18 and 19).* The mean readings for the observed partial pressure of oxygen in the chamber was 1.13%, range (1.04 - 1.23). When comparing the mean reading of the set pressures which was 1% to the observed over the 24hour period using paired t-test; both means were similar with p value = 0.006 which was deemed sigificant-($p<0.05$).

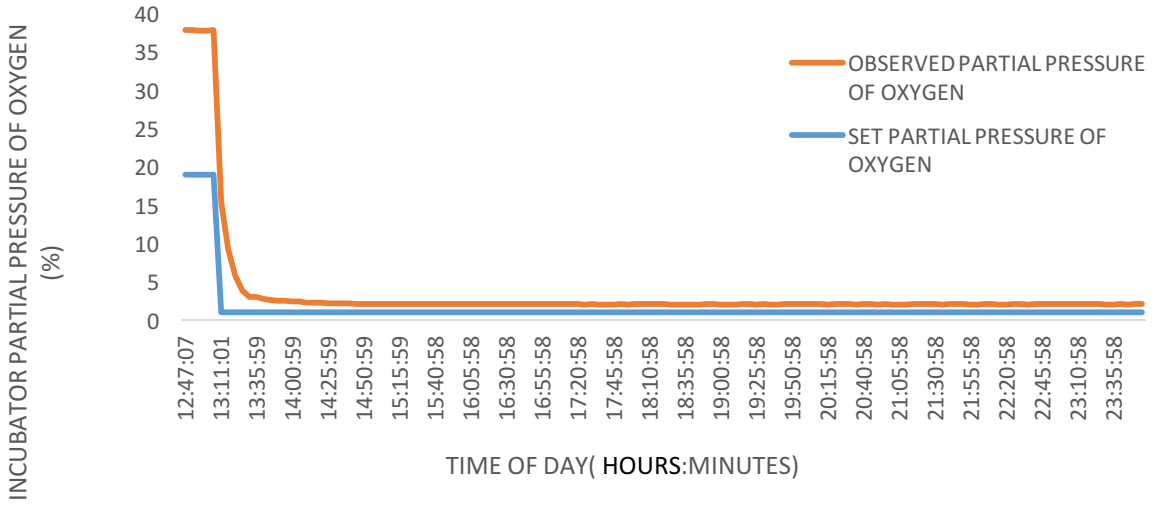

Figure A26- *Showing incubator oxygen concentration over the first 12 hours with the trend of the observed and the set partial pressure of oxygen in the incubator chamber. It shows that the set and observed measure were the same. Paired t-test showed $p<0.05$ which was significant.*

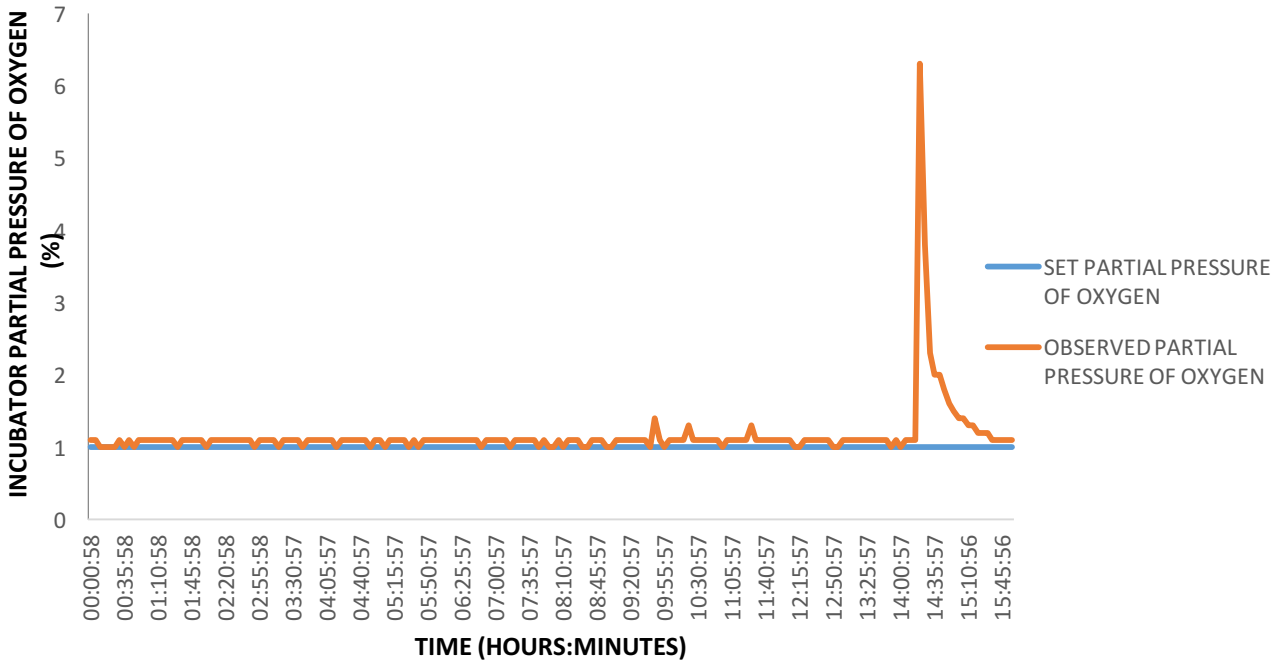

Figure A27- *Showing incubator oxygen concentration over the next 12 hours with the trend of the observed and the set partial pressure of oxygen in the incubator chamber. Please note the spike seen here was at the end of the experiment when incubator chamber door was opened.*

CONCLUSION.

It is clear from the above results that the set reading for the partial pressure of oxygen in the incubator chamber is not significantly different from the actual observed readings. This shows that the environmental hypoxic condition in the chamber can be reliable and the same can be concluded for the Galaxy R- oxygen incubator used for the hypoxic cell culture in this study. We also conclude that these conditions therefore are reproducible.

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